IMMUNOHAEMATOPOIETIC STEM AND PROGENITOR CELL TRANSPLANTATION

A THIRTY YEAR PROSPECTIVE AND SYSTEMATIC RESEARCH INVESTIGATION

BY

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Submitted in fulfilment of the DSc in Medical Science at Stellenbosch University

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SUPPORTING CURRICULUM VITAE
DECLARATION

I, the undersigned, hereby confirm that work contained in this dissertation is my own contribution. It has not previously, in its entirety or in part, been submitted at any University for a degree.

[Signature]

PETER JACOBS

[Signature]

DATE
DEDICATION

This thesis recognises the unfailing support of my wife and our two sons Sean Keiran and Wayne Clinton during three decades of still ongoing academic commitment.

It additionally honours the memory of our parents whose selfless sacrifices made possible a professional career.
ACKNOWLEDGEMENTS
Stellenbosch University for the honour bestowed by encouraging continued scientific, academic and intellectual commitment initially as the Honorary Professor of Haematology in 1995. Approximately a decade later to Foundation Professorial Headship of the newly established Division of Clinical Haematology within the Department of Internal Medicine - Faculty of Health Sciences and Tygerberg Academic Hospital.

To mentors, peers and role models is recorded, with the deepest possible respect, gratitude for instilling into me those principles of integrity, dedication, honour and humility that are inviolate in teaching students, training postgraduates and undertaking research. Without these ever-present unique examples no personal or other standards can be judged subjectively or measured objectively.

None of this would have been possible were it not for that first stimulus of basic scientific training that started during the student years. Early growth then nurtured as Clinical Tutorial Registrar at the University of the Witwatersrand in the Department of Internal Medicine by Professor Thomas H Bothwell. It was his uncompromising striving for perfection that has remained a beacon for half a century. Experience was accumulated through the opportunity as Eli Lilly Travelling Scholar and National Institutes of Health Senior Research Bursar to work with Professor Clement A Finch in his Division of Haematology at the University of Washington. There was kindled, at this time, an interest in stem cell physiology and the newly emerging application of these principles to bone marrow transplantation by contact with the legendary Nobel laureate Professor Edward Donnall Thomas and his Seattle team.

A seminal event was the privilege of appointment as Foundation Professor and Head of the new autonomous Department of Haematology at the University of Cape Town and Groote Schuur Hospital. With the recent and successful first human heart transplant by Professor Christiaan Neethling Barnard the environment in the medical school, strongly supported by staff at every level, was optimally conducive to the introduction - and subsequent development - of haematopoietic autologous and allogeneic grafting.
To many associates, and particularly the Haem Team that exists in the original unchanged format of a multidisciplinary group to this very day, belongs much credit for the pride of place that these procedures enjoy currently in this country. The example set by professional nurses, medical laboratory technologists and support staff - whether these were secretarial or janitorial - ensured that over decades one has been kept ever sensitive to the guiding principle – that of remaining a perpetual student.

Sister Lucille Wood as colleague and scientific collaborator over three decades: Mrs Christine Dölling and Miss Natasha Trueman as librarian and typist devoted long hours to cataloguing all the voluminous data and reliably typing the manuscript: sincere thanks to these three research co-workers.

Penultimately special collegial indebtedness to Patrick Bouic for being willing to promote this doctoral dissertation jointly with Stephen Hough. These two distinguished Professors have provided support and carefully considered advice during all phases from concept to submission. They have additionally guided the various stages through amendments required for approval by the protocol evaluation committee chaired by Professor Johann Schneider. This ethos reflects all that is good in a true university system and is unreservedly saluted.

Finally the raison d'être for each and every aspect of this all too short journey will forever remain the courage and fortitude of our patients and their families. These times were often difficult and carried with them great sadness but were balanced in others by modest success. This cumulative experience remains the powerful stimulus to keep alight that quest for new knowledge. It is a fervent hope that those who we serve will derive the benefit they expect from dedicated doctors and nurses in general, haematologists in particular and specifically the transplant community worldwide into whose midst we have the great honour of being accepted.

What greater reward or sense of fulfilment could any individual aspire to?
CHAPTER 1

EXPLANATORY INTRODUCTION
Orientation

An abortive attempt, that involved myself during the nineteen sixties in Johannesburg to allograft a medical student with refractory bone marrow aplasia, failed. This was primarily due to lack of understanding tissue-typing and the fundamental principles defining graft composition as well as recipient conditioning. Subsequently interest in the area was awakened during tenure of a Haematology Fellowship with Professor Clement A Finch at the University of Washington in Seattle as an Eli Lilly travelling scholar and National Institutes of Health senior research bursar. During this period one was privileged to benefit from stimulation and encouragement of the already legendary Professor Edward Donnall Thomas – subsequently Nobel laureate - and his team: an association that remains intact to this day. The opportunity to pursue a systematic study of stem cell immunobiology, as the basis for participation in the rapidly expanding field of transplantation, came about by the honour of appointment as Foundation Professor of Haematology at University of Cape Town and Groote Schuur Hospital. Reciprocally a commitment was given, as the first incumbent, to establish this area as the future direction for the new department. It is also appropriate, and a distinct honour, to acknowledge many distinguished friends and colleagues - both local and international – who to greater or lesser extent shared in this vision.

All those quiet and unobtrusive voices – now as much as then - unhesitatingly promoted this growth point as a realistic long term project. The basis was recognition of the fertile environment created by the first human heart transplant carried out by one of South Africa's greatest sons - Professor Christian Neethling Barnard.

Organisational philosophy

From inception one fundamental scientific principle, reinforced throughout postgraduate training, has been scrupulously adhered to. Thus each individual step logically consecutive, tested and consolidated before embarking on the next segment of the work. For example in vitro and animal studies were completed in the laboratory prior to evaluation in patients. This observation is made only to explain the way in which reports and presentations, peer reviewed en route to publication of outcome in first line journals, are organised in this thesis.
Furthermore it is noted that careful consideration has been given to all the Stellenbosch University policy and procedures for postgraduate students, including those referring to plagiarism, thereby facilitating a declaration that the concepts, experimental design and laboratory as well as human investigations being my own.

**Objective - Title and Scope**

This prospective work, approved by Stellenbosch University Protocol Evaluation Committee, can be somewhat artificially presented in two, but inseparable, components.

The first is participation in global or international transplantation programmes. This ongoing exercise explores the pathophysiology of bone marrow failure by collaborative studies such as those committed to understanding aplasia°. Using this autoimmune model to interactively work within European°-4 and American°6-8 groups constantly aiming to translate progress to our unit. To ensure that growth points actually included South Africa - to seek and have approved - registration of every outcome by sustained audit and accreditation°9. These mechanisms remain unbroken to the present time and underscore compliance with worldwide ethical and research principles.

Simultaneously to define a role for the interventions in haematologic malignancies such as leukaemia°7, lymphoma°8,°11 and myeloma°12,°13,°14 including amyloidosis°3 and wider afield to immunodeficiency and other disease categories including solid tumour oncology.

The second to cultivate a research environment supported, on merit and productivity, by University grants and investigator driven projects within the Medical Research Council as well as the National Cancer Association. The consistent theme remained documentation of haematopoietic recovery coupled with systematic exploration of cellular and humoral phenomena underlying graft failure and the various forms of injury to keratinocytes, enterocytes and biliary endothelium°15,°16,°17.
Methodology required careful selection of a suitable animal model in which to set-up automated blood counting. Thereafter to apply the standardised protocols to quantitative and qualitative characterisation of the mononuclear suspension from harvested marrow ensuring that these could be applied to our patients\textsuperscript{18}. To define, by introducing suitable immunophenotyping, the composition of this population and document long-term colony repopulation potential via clonogenic assay.

Blood and marrow reconstitution was standardised to use two inbred rabbit strains for creation of a model recapitulating the clinical graft-versus-host disease syndrome. Once secure to sequentially explore means for prevention and treatment with external quality control maintained by close interaction with the Basel team\textsuperscript{19}.

Based on preliminary experimental haematology there emerged further collaborative studies with the Swiss documenting benefit for administration of cyclosporin - A to the recipient. The end result was only partially successful. So to further improve outcome established with scientists from Oxford a joint programme to systematically investigate and subsequently describe exposure of the incoming graft \textit{ex vivo} or in-the-bag to the anti-CD 52 Campath series of monoclonal antibodies\textsuperscript{20}.

It is specifically reiterated that each and every step complied with local and international ethical and research guidelines including those from the Declaration of Helsinki. Also, that all patient studies were not only on approved protocols but individuals gave written fully informed consent: a practice still in force.

Experience accumulated over the three decades of consecutively completed, analysed and reported cases was systematically assembled. This imperative defined the success of developing a research group to actively participate in the utilisation of immunoohaematopoietic stem cell transplantation immunology and technology. From modest beginnings, for more than a decade in a solitary centre, there followed dissemination of these procedures throughout the country.
The second unit started with Professor Thomas H Bothwell at the University of Witwatersrand some 15 years after consolidation of the Cape Town programme. Further expansion necessitated establishment of a South African Bone Marrow Registry\textsuperscript{21} to accommodate the needs for location and recovery of grafts from matched-unrelated volunteers\textsuperscript{20,21,22}. Ongoing activity explores and extends the scope with a shift in emphasis from replacement to restorative medicine\textsuperscript{23}.

Concurrently to encourage endeavours in countrywide cooperation by activity reporting\textsuperscript{24}. This goal is envisaged as a first move to generating national collaborative clinical trials. These would have the importance of taking into account particular constraints existing in under resourced facilities with the compounding unique challenges of an escalating Acquired Immune Deficiency Syndrome epidemic\textsuperscript{25,26,27}.

\textit{South Africa in context of the first world – 1970 scenario}

Throughout this country eminent physicians practised haematology with the discipline located primarily in service pathology laboratories. One consequence was that local knowledge was not collected and published so that a reliable database could not exist. Understandably there resulted a general under-appreciation of the way in which more structured departments, that inseparably linked clinical and pathology components including immunology with blood transfusion technology\textsuperscript{28,29}, were evolving. Thus little uniformity existed about how well recognised and often lethal entities, with the prototype being bone marrow failure described as severe acute aplastic anaemia, were diagnosed and much less treated! There were areas of considerable expertise exemplified by nutritional anaemia studied by Professor Jack Brock and his associates at the University of Cape Town, iron metabolism and vitamin B\textsubscript{12} with Professor Thomas H Bothwell and Professor Jack Metz respectively in Johannesburg, platelet physiology and pathology in Bloemfontein. Additionally had been the very successful integration of all these aspects under the broad ambit of haematology by Professor Harry Greig in Durban. However, strikingly lacking from this landscape, was any cohesive effort to bring together those components needed for stem cell transplantation.
Additionally there was the unusual situation where many of the haematologic malignancies such as lymphoma and myeloma were treated primarily in radiotherapy without the benefit of strong multidisciplinary or combined clinics. The challenge was to harness these vibrant pockets of expertise\textsuperscript{30,31,32} having many quite different ideologies and forge a new dispensation focusing on stem cell biology. Many individuals shared in getting to this goal and these contributions recently paid tribute to\textsuperscript{33}.

**Professor Edward Donnall Thomas and the Seattle connection**

The above situation was highlighted during haematology fellowship with Professor Clement A Finch at the University of Washington that brought one into contact with the new and stimulating concept of bone marrow transplantation. The focal point was a team led by the already legendary Dr Edward Donnall Thomas — subsequently Nobel laureate — that had developed a sound understanding of how blood forming stem cells could be transferred between donor and recipient using a canine model\textsuperscript{34-36}. Concurrent technology was expanded through the use of continuous flow blood fraction separation, exploration of the cellular content and function of the harvested marrow\textsuperscript{37} with definition of the stem cell immuno-phenotypically using flow cytometry. The indications for these procedures emerged from two landmark publications in the New England Journal of Medicine so that clinical bone marrow transplantation started to attract ever-increasing worldwide recognition of its value\textsuperscript{34-38}. At the same time the unwanted consequences of the immunologic attack on a number of recipient organs and tissues, ranging from graft rejection to the dreaded entity of acute and chronic graft-versus-host disease, became recognised\textsuperscript{39,40}.

Early on complications of marrow harvesting from the donor became a focus of attention and comment on an all too frequently overlooked somewhat prophetic analysis\textsuperscript{41}. The enormity of the side-effects in the patient started to draw attention to the importance of quality of life and appreciation, it is interesting that survivorship was already an emerging consideration\textsuperscript{42-43}. It was into this worldwide transplantation community that one ventured recognising not only the value of a multidisciplinary approach but, specifically, how important and privileged it would be to have a chance of participating in existing registries with access to knowledge available from collaborative studies.
Many of these possibilities came to fruition with the great honour conferred by appointment as Foundation Professor of Haematology at the University of Cape Town. It was now possible to commit the new Department in the Faculty of Medicine to the experimental studies and project subsequent translational research calculated to responsibly move these procedures into clinical practice.

It is a matter of further privilege that, throughout these years, one has enjoyed the constant and personal encouragement of Professor Edward Donnall Thomas and the team at the Fred Hutchinson Cancer centre at the University of Washington. This is affectionately referred to as the Seattle connection and remains active at the present time.

*International collaboration over three decades*

From the earliest days of exploring the unique characteristics of the immunohaematopoietic stem cell two priorities were evident.

One was a need to capitalise on clues from cytomorphologic characteristics of tumour cells and, to this end, chance to participate in the lymphoma classification project in association with Professor James Armitage in the medical centre at Omaha Nebraska. This has had three important influences over the last 20 or more years. Thus there has been a constant and ready contact with one of the most distinguished and active transplantation groups in the world. Another to share in a series of collaborative investigations exploring geographical differences in the lymphomas and this is actively ongoing, including exchange and training programs for PhD students at that centre. A third to critically access to the particular expertise in the management and harvesting of these populations in general but with particular focus on the subsequent utilisation as an integral component of treating tumours of the lymphoreticular or immune system. This lively exchange of information is reflected in 25 years of collaborative existence of the South African Lymphoma Study Group.

The other was an acute awareness that accountability for every consecutive patient treated, including outcome analysis, mandatory in the maintenance of credibility and standards.
The privilege of participation in the lymphoma group of the European Organisation for Research and Treatment in Cancer has, to this day, maintained a critical reference point for protocol management. Recently these activities have been enhanced by interaction with the German Hodgkin Study Group culminating in the first joint meeting. To maintain the transplant focus there is active involvement with the European Group for Blood and Marrow Transplantation in both the chronic leukaemia and the acute leukaemia working parties. In this latter cooperation it is notable that this remains the only team listed as a transplant centre in this country.

As part of this broadly based and innovative landscape to maintain close relationships with other study groups in order that the perspective for all these procedures can be balanced against alternative treatment options. Thus association with the Myeloma Trialists Collaborative Group as well as the International Myeloma Working Group have proven valuable in this role.

*Local research and development in the corresponding period*

The historical and scientific perspectives spanning 35 years of prospective and structured research, where one project was the building block for the next stage, are a matter of record and the basis for this doctoral dissertation.
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BONE MARROW FAILURE:
PATHOPHYSIOLOGY AND
MANAGEMENT

Peter Jacobs, MD, PhD
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Mosby
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FOREWORD

The past 25 years has seen tremendous growth in our understanding of the way in which bone marrow performs. Hematologists have made great strides toward elucidating the cause of many bone marrow disorders, allowing the development of more appropriate therapy. And, of course, the last 25 years has seen the development and refinement of bone marrow transplantation, which for the first time has allowed us to cure some of these patients. Professor Peter Jacobs has long been on the cutting edge of this exciting specialty. In the current issue of Disease-A-Month, Dr. Jacobs provides us with a comprehensive view of the hematologic issues involved in bone marrow failure. His ability to translate the findings of experimental hematology into understandable concepts and to translate the relevancy of these concepts as well has gained Dr. Jacobs a worldwide reputation. I know that you will refer to the current issue again and again in your practice. We are fortunate to be the recipients of Professor Jacobs' expertise.

Roger C. Bone M.D.
Editor
BONE MARROW FAILURE:
PATHOPHYSIOLOGY AND MANAGEMENT

ABSTRACT.—Morphologically, bone marrow is made up of a relatively mature but heterogeneous population, fueled by a tiny pool of microscopically unrecognizable stem and progenitor cells. This complex tissue has the responsibility of maintaining our hematopoietic and, to a large extent, immunologic integrity, both of which are indispensable for health and, indeed, survival. Perhaps not surprisingly, bone marrow is the target of genetic, autoimmune, and environmental insults. Although robust, it has only a limited number of responses, one of which is reduction in cellular output, sometimes with superimposed qualitative abnormalities, and this is defined as bone marrow failure.

Bone marrow failure is a diverse entity but can be logically explained and classified on a pathophysiologic basis. Thus the major recognizable categories of bone marrow failure are congenital and acquired defects. Each of these is subdivided according to the number of cell lines involved, over and above which the severity of the damage will determine reversibility. In each case, the natural history dictates management, and this ranges from short-term growth factor support to biologic immune response modulation and finally to bone marrow transplantation.

In the past, many clinicopathologic variants of bone marrow failure were described, although their etiology was obscure and effective therapy was unavailable. This changed dramatically, however, when experimental hematologists, using radiobiology models, uncovered the dynamic nature of blood formation. Cardinal observations included the way in which spontaneous recovery followed irradiation, the central role played by pluripotent stem cells, and the integral participation of stroma in modulating this entire process. Understanding was refined once bone marrow cultures became available while, in parallel, the use of inbred mouse strains launched the era of allogeneic transplantation.

These approaches were combined, and the broad principles that govern basal or constitutive production emerged. Stem cells, with their characteristic commitment to self-renewal, exist at the apex of a hierarchy and generate a tier of proliferating progenitors that, in turn, give rise to a large postmitotic compartment of precursors that mature into distinctive myeloid and lymphoid lineages. The reserve potential is enormous, and output can be induced to meet even greatly increased demands. These events reflect the interaction of growth factors with a balancing set of negative regulators. The link between such diverse functions resides, to a large extent, in accessory cells and matrix geographically organized in what is now described as the hematopoietic inductive microenvironment.

Many details of these meticulously orchestrated processes are obscure. For example, how are adhesion proteins, expressed on vascular endothelium, subtly altered to facilitate release of mature cells into the circulation? Enigmatic also is the way in which membrane antigens change during recovery from chemotherapy or in response to growth factors, so that early progenitors flood the circulation and can be collected for subsequent autografting.

In spite of our newfound knowledge, patient management is often empiric. To illustrate this point, consider the inherited Josephs—Diamond—Blackfan anemia or global aplasia described by Fanconi, in which undoubted benefit—indeed cure—is possible with the nonselective transplantation of marrow. Our understanding of this process is rather crude and is a far cry from the exquisite specificity with which a solitary molecular defect, once defined, can be repaired using gene therapy.

Patients with acquired lesions are little better off. Some, such as those with idiopathic pure red cell aplasia, enjoy spontaneous remission, whereas others require varying degrees of pharmacologic immunosuppression. Perhaps more frustrating are the many cases of unexplained loss of hematopoietic tissue. It has been suggested that such individuals have a genetically fragile marrow that collapses when subjected to...
a relatively minor environmental insult and that possibly the collapse is acting through an immunologic mechanism. For want of a precise molecular diagnosis, the same relatively unsophisticated therapeutic approaches are used in these patients, albeit with substantial success. In this context, allogeneic transplantation is limited by rejection, whereas stable engraftment may be hampered by the not insignificant incidences of morbidity and mortality that result from graft-versus-host disease. Unfortunately, even this option is not always available, and alternatives such as antilymphocyte globulin, which do bring about quantitative responses, may be followed by the later development of myelodyplasia or acute leukemia. These obvious shortcomings prompted investigators to study closely the convoluted trail that leads back to faulty DNA to allow more exact intervention.

Against this background, there arises the question of how to structure a pragmatic and relatively uniform approach to the care of patients with bone marrow failure. Logically, treatment modalities should center on the currently understood model of hematopoiesis, in which lesions can arise in early stem cells, resulting in aplasia. This is in contrast to the damage of already committed progenitors in which, at least initially, single lineages are affected. Within each category, the defect may be inherited or acquired. Approached pathophysiologically, an algorithm for treatment evolves naturally and advocates simple support for those in whom spontaneous recovery is predicted, although this increasingly incorporates the use of recombinant human growth factors or interleukins, which are often given together. For irreversible damage, bone marrow transplantation is currently the treatment of choice. If this option is lacking, immunosuppressive regimens are effective, with the caveat that complications must be anticipated. The future is somewhat brighter, with the promise that more precise molecular diagnosis is, in many cases, well within our grasp. Armed with such vital information, gene therapy becomes a realistic possibility; precedent already exists in which adenosine deaminase deficiency, an otherwise lethal immunologic disorder, can be precisely corrected.

IN BRIEF

Like so many things in life, health is largely taken for granted, and this includes having normal blood, with its impressive range of functions. Consequently, bone marrow failure is a traumatic event, reflected in diverse clinical and hematologic syndromes. In some cases, bone marrow failure is inherited and is present in childhood, as opposed to acquired variants, which usually manifest in later years. In both situations, red and white cells, or platelets, may be reduced singly or in combination, often with superimposed qualitative abnormalities. Symptoms of one or the other cytopenia are common, so that relevant investigation and rational management require an appreciation of the etiology and natural history of cytopenia. A minority of patients recover spontaneously, although more typically, there is relentless progression, often extending to previously uninvolved lineages and leading to substantial morbidity and mortality.

The paradigm is severe acute aplastic anemia. This is a semantically incorrect synonym for global marrow failure but, nevertheless, is a suitable point from which to chronicle those many dynamic events concealed behind the bland histology of the trephine biopsy. Microscopically, we recognize only the mature precursors, whereas the hematopoietic stem and progenitor cells from which the mature precursors arise are nondescript. Furthermore, and for far too long, the complex microenvironment in which blood formation takes place was assigned a subsidiary role, being largely conceived of as providing mechanical support alone. All this has changed with the appreciation that these two populations are inextricably linked through an ever-widening range of growth-facilitating peptides that maintain physiologic or constitutive levels of production, which can, in times of need, be induced to expand dramatically. The balance necessary to meet homeostatic demand precisely for any one or all lineages is meticulously orchestrated by swift and highly selective responses to a series of stimulatory or inhibitory molecules. These ligands attach to cell-surface receptors that, once activated, transduce their signals rapidly through substrates in the cytoplasm to the nucleus, with the terminal one in the chain being described as a transcription factor. These, in turn, switch on or off genes that initiate responses as divergent as entering the cell cycle to proliferate to being diverted to programmed death, the other name for which is apoptosis.

In the last 25 years, such ‘new’ hematologic has spawned a whole new terminology and is, at first sight, daunting and appears remote from patient care. Nothing could be further from the truth. Understanding these principles has increased the basis for management. Unfortunately, current approaches are relatively crude, ranging from the administration of recombinant growth factors to bone
marrow transplantation. Nevertheless, it is from here that gene manipulation and insertional therapy, with their exquisite specificity, are likely to accelerate our understanding and improve patient outcome in the immediate future.

A historical perspective on bone marrow failure is provided by the observation that death in animals undergoing lethal radiotherapy was prevented either by seeding the animal with hematopoietic tissue or when autologous reconstitution took place from a shielded femur or spleen, thus establishing the existence and migratory nature of a multipotential stem cell. At the same time, tolerance by the recipient to donor marrow was systematically explored using total body irradiation and immunosuppressive drugs. This culminated in the monumental studies by Dr. Thomas and his associates at Cooperstown, who showed the feasibility of allografting and autologous transplantation with cryopreserved cells, but who provided the insightful caveat that graft-versus-host disease would emerge as a formidable problem. Predictably, early experience was discouraging because the significance of matching at the major histocompatibility complex was not yet appreciated. Nevertheless, the stage was set, and the first successful procedures were performed approximately 25 years ago in children with immunodeficiency, and immediately thereafter, the procedure was successful also in patients with acute leukemia. Currently, this procedure is the preferred form of management for many diseases and, notably, for bone marrow failure. It is the culmination of these experimental and clinical studies, supplemented by experience derived from clonogenic and long-term culture studies, coupled with the availability of recombinant growth factors, that has led to our present understanding of cellular output.

Blood production is achieved through a continuum of events, but it is convenient to consider those steps that transform unrecognized ancestors into a variety of mature progeny. On this basis, the pathogenesis, natural history, classification, and management of rather different disorders that are broadly grouped together as bone marrow failure are described.

Stem cells are heterogeneous, but they are functionally defined by an ability to sustain comprehensive lymphohematopoiesis. Their first division initiates the process of differentiation, contributing a daughter to maintain the integrity of this compartment and a progenitor that has lineage commitment and proliferative potential but with a reduced marrow repopulating capacity. Stem cells can be studied in a variety of culture systems, but none of these reliably reproduces the conditions that prevail in the intact bone marrow, and they are therefore inadequate to provide precise characterization. Morphologically, the stem cells bear a close resemblance to small lymphocytes, with typical expression of the CD34 antigen. An additional marker is the intensity of staining with dyes such as rhodamine B.

However, probably because of the low frequency with which they normally exist, the goal of isolating stem cells in large enough numbers for clinical use is, at present, tantalizingly just out of reach.

Growth factors initially attracted attention as stimulatory peptides. However, and predictably, molecules that exert the opposite effect on hematopoiesis, exemplified by the tumor necrosis factor, have since been identified and designated as negative regulators. Homeostasis is maintained by ligands in both categories attaching to their cognate transmembrane receptors, which, in turn, serially alter with the degree of differentiation. Such engagement is followed by a series of cytoplasmic events cascading down incompletely characterized pathways to transduce the external signal toward the nucleus. These events culminate in the generation of molecules or transcription factors that have a particular configuration or motif whereby they attach to specific genes whose function they then modify. As a result, cellular response appropriately ranges from entering the cycle to proliferate, mature, alter its function, or undergo cell death—all in accordance with basal requirements or as an adaptation to environmental stress.

The inductive microenvironment finally came into its own after cross-transplantation studies between inbred mouse strains that have a common phenotype of refractory anemia, absence of mast cells, and changes in skin color. These studies established that mutations involving quite different genes had the same result. In one, the product of the c-kit oncogene was not expressed, but the hematologic defect could be corrected by providing its normal counterpart. In the other, a mutation at the Steel locus prevented synthesis of the stem cell factor, resulting in a microenvironment that was incapable of supporting hematopoiesis. This type of complex interaction could not be unraveled in vivo, but with the availability of long-term cultures, it became clear that there exists a major network of accessory cells, ranging from adipocytes to vascular endothelium, and these synthesize a vast array of metabolically active products, many of which are stored or presented in an associated extracellular matrix. Conceptually, niches are present as highly specialized microgeographical areas, where adhesion molecules facilitate binding and correct alignment between stroma and progenitors. Although simplified, it is clear that these three major components constitute a single functional unit charged with the physiologic regulation of blood formation, and understanding this is helpful in our consideration of the syndrome of bone marrow failure.

Inherited defects may remain confined to a single lineage, as in Josephs-Diamond-Blackfan anemia and Kostmann's neutropenia. Alternatively, they may evolve, giving rise to global damage. White cells are affected initially in the Swachman-Diamond syndrome, and reticular dysgenesis occur or platelets are affected in those with
amegakaryocytic thrombocytopenia with absent radii. Conversely, all
the elements may be simultaneously lost in autosomal recessive aplas-
ia or its Esten–Dameshek variant. A similar sequence occurs in pa-
tients with dyskeratosis congenita. In the first example, as originally
described by Fanconi, there are distinctive clinical features and char-
acteristic chromosomal breakage, which is thought to reflect homozy-
gosity for genes giving rise to bone marrow failure. An interesting pos-
tulate is that the heterozygous state may predispose patients to he-
matopoietic failure after minimal superimposed environmental in-
sult.6

Acquired lesions are often reversible, resulting from exposure to a
variety of drugs and toxins and, although immune mechanisms have
been incriminated, most cases remain unexplained. Unfortunately,
and with surprising frequency, a previously healthy individual may
develop idiopathic pancytopenia as a result of irreversible loss of mar-
row precursors; we recognize this entity as severe acute aplastic ane-
mia. However, this is a misnomer because loss of red cells is but one
component of the much more widespread pathology.

A sensible algorithm when confronted by a patient with single or
multilineage cytopenia, particularly when it is severe and irrespec-
tive of symptoms, is to ensure that an accurate and, if possible, etio-
logic diagnosis is established immediately. This will depend on a me-
ticulously taken history to uncover both similar examples in the pa-
tient’s family and any exposure to drugs, toxins, or medications and
is followed by a careful physical assessment. Full blood count is fol-
lowed by bone marrow examination that should include cytogenetic
and, where appropriate, specialized culture studies. It is mandatory
to recognize and withdraw offending myelotoxic agents and treat as-
associated medical conditions.

The natural history of the disorder will determine outcome. How-
ever, because this cannot always be predicted, there is much to rec-
ommend early referral to an experienced multidisciplinary manage-
ment team so that the appropriate level of supportive care can be
provided until spontaneous remission takes place and the danger is
past. All too frequently, lack of judgment leads to well-intentioned
under or overtreatment, both of which have avoidable complica-
tions.

Irreversible aplasia is a particular problem that should be identi-
fied swiftly to avoid sensitization by transfusion of blood and related
products. Compatible sibling bone marrow transplantation is the
treatment of choice. Lack of a suitable donor is an indication for bi-
ologic immune response modulation or immunosuppressive regi-
ments, although both produce variable responses, and long-term out-
come remains uncertain. For this reason, recourse to family mem-
bers or matched unrelated marrow grafts, using the services of inter-
national registries, is an expanding option.

In the short term, refinements in allogeneic transplantation are
needed to reduce rejection and abolish graft-versus-host disease. To
this end, increasing the purity and yield of stem cells, exploring um-
bilical cord blood as an alternative source, and expanding stem cells
in suspension culture using ex vivo methods and stimulating them
with a variety of growth factors are clinical realities. In the longer run,
hope rests on the application of cellular and molecular biologic tech-
niques to identify individual defects that would then become ame-
nable to specific correction through gene transfer. Advances in these
areas have been impressive, and with the ever-increasing rate at
which DNA technology is being translated into the clinic, we hope
that the next decade will see definitive diagnosis and cure emerge
for many of our patients with bone marrow failure.
Peter Jacobs was educated at Prince Edward School in Salisbury, Southern Rhodesia. He then spent 6 years as a medical laboratory technologist before beginning to study medicine at the University of the Witwatersrand in Johannesburg, where he won a number of scholarships and graduated with class medals in medicine and surgery. From an early stage, Dr. Jacobs was attracted to research and studied iron metabolism as a Council for Scientific and Industrial Research bursar. He received both Eli Lilly and NIH Fellowships from the University of Washington, where he also served as Director of the Division of Hematology in the Department of Laboratory Medicine. During this period, Dr. Jacobs' area of interest shifted to the study of hematopoiesis under the influence of Dr. E. Donnell Thomas and his bone marrow transplantation group. Shortly after returning to South Africa, Dr. Jacobs was appointed Foundation Professor of Haematology at the University of Cape Town. During the last 25 years he has directed a team that continues to study systematically different aspects of hematologic malignancy and bone marrow failure, using clonogenic assays, long-term culture systems and, increasingly, cytogenetic, cellular, and molecular biologic techniques. From this laboratory base, multidisciplinary management strategies have been developed that encompass the use of hematopoietic growth factors, allogeneic transplantation, and autografting with stem cells derived from marrow or peripheral blood stem. Dr. Jacobs is a Fellow of the American College of Physicians, the College of Medicine of South Africa, the Royal College of Physicians, the Royal College of Pathologists, and the Royal Society of South Africa. He is a member of numerous other distinguished scientific groups as well and serves on the editorial boards of several international journals. He has traveled widely as a visiting professor.

BONE MARROW FAILURE: PATHOPHYSIOLOGY AND MANAGEMENT

THE PHYSIOLOGY OF HEMATOPOIESIS

INTRODUCTION

Students at all levels, including hematologists, have their first real contact with medicine in the time-honored courses of gross anatomy and physiology. Microscopy is then used to disclose the way in which cells are arranged into tissues, and minute detail is revealed ultrastructurally. These inert sections on slide preparations are brought to life by showing how their component enzymes and proteins function biochemically. In recent years, technological advances have unraveled many interactions, and the modern graduate is increasingly concerned with molecular regulating mechanisms, such as those for growth and apoptosis; the latter is known as programmed cell death.

These observations apply equally to the hematopoietic system, which, understandably and for many years, was described largely in terms of increases or decreases in recognizable mature elements in the blood or bone marrow. This began to change more than 20 years ago when the dynamic nature and output of this organ, which is approximately the size of the liver, was elegantly restated (Fig. 1). Tantalizing questions arose about its potential capacity to respond to bodily needs for each of the major cell types, a visionary role for the vasculature was postulated, and the existence of chemotactic signals was anticipated as the mechanism for cell release into the peripheral circulation. Once radioactive isotopes became available, it was possible both to image and to study kinetic events, making it possible to understand better the changes shown in the trephine biopsy and what they meant in functional terms.

Almost immediately, bone marrow failure, which was already recognized as an important topic in clinical medicine, came under intense scrutiny, with etiologic classification being linked to pathogenesis. Further insights into physiology and its disturbances were gained from carefully conducted experiments in animal models, including seminal studies of syngeneic and allogeneic transplantation. Subsequently, the availability of clonogenic assays brought new in-
FIG 1. In lymphohematopoiesis, a small population of stem cells divide. This pool is sustained while contributing a progenitor that has a lesser capacity for self-renewal but that increases lineage commitment. The major characteristic of this progenitor is proliferation.

sight into the response of early progenitors to growth factors, and long-term marrow cultures revealed the vital interdependence between these cells and the supporting microenvironment or stroma. Molecular biologic techniques are showing the way in which stimulatory or inhibitory molecules react with membrane receptors and initiate signals that are transduced to the nucleus and activate genes whose products bring about the specific homeostatic function required.

From this physiologic basis has grown an appreciation that hematopoietic failure can result from constitutional defects, whereas similar syndromes may be acquired through environmental injury acting in isolation or perhaps as a second insult in a genetically predisposed individual. Furthermore, there is greater awareness that in any situation, qualitative deficiencies may be superimposed on these quantitative changes, although the latter are generally easier to detect.

Perhaps predictably, a whole new terminology has evolved to describe modern-day understanding of blood formation. This lexicon can be confusing and at first sight so daunting that many clinicians shun away from the topic, believing that such minutiae can have little direct application to their practice. Nothing could be further from the truth. Indeed, there is an ever-increasing translation of experimental results into the more rational management of patients with marrow failure. The latter entity is surprisingly common, so that one or another variant is likely to be encountered, not infrequently, by the pediatrician, general practitioner, or primary care physician. Thus it is important that the mystique still surrounding this rapidly evolving area be dispelled by describing, in simple terms, a model that links these various phenomena. It will then be easier to appreciate etiologic mechanisms and anticipate the natural history as a basis for selecting appropriate therapy. Notwithstanding such an approach, treatment often remains empirical, ranging from the administration of recombinant human growth factors through immunosuppression to allogeneic grafting.

The challenge for this monograph is threefold. The first part of the challenge is to assemble what is currently known about the anatomy and physiology that regulate marrow output. The second challenge is to provide a practical approach to the classification of the syndromes that are of clinical importance. Finally, the last challenge is to consider the mechanisms that underlie the failure of the bone marrow to meet homeostatic requirements as a basis for contemporary management.

OVERVIEW OF THE HEMATOPOIETIC SYSTEM

Histogenetically, fusion of sperm and ovm result in a totipotential stem cell, from which, during development, different organs evolve through their own unique pluripotential counterparts. The untold steps en route to the recognizable embryo, although many continue to attract attention, remain shrouded in mystery. Some glimpses into the complexity of achieving a defined form are reflected in the examination of the Drosophila species, in which homeotic genes control the sequential assembly of body segments in a way that is probably analogous to what occurs in humans. Thus morphologically recognizable blood islands appear first in the yolk sac and move transiently to the liver and spleen, eventually localizing to the medullary cavities of the skeleton.

The details of how this migratory pattern is controlled are uncertain, but precedent from the fruit fly suggests a central role for homeobox genes that additionally encode transcription factors for many hematopoietic functions, including immunoglobulin synthesis and lineage commitment. These, like traditional oncogenes, are clearly crucial for normal organogenesis, but dysregulation through translocations has profound effects, including the development of tumors or leukemia. Precisely how these events result in neoplasia, with the characteristic uncoupling among differentiation, proliferation, and
maturation, is not clear because there are normally check points on intracellular events, such as cell cycling. For example, control exists at both the G1 to S transition and also at entry to mitosis, with the enzymatic activity of the regulatory protein kinases vested in cofactors, known as cyclins because of the way in which their concentrations rise and fall throughout this event. What does emerge, however, is that from fertilization to delivery, each step in the development of the blood-forming system is genetically determined, and many of the same mechanisms continue to operate throughout life. It follows that disruption or imbalance of these modulators may predispose or actually lead to syndromes at the opposite end of the spectrum—bone marrow failure or hematologic malignancy.

Anatomically, the marrow, when seen in histologic section, is deceptively simple, with its high level of activity difficult—even impossible—to appreciate. At this level, prominent features are the bony trabeculae with their associated osteoblasts and osteoclasts, blood vessels that vary in size, and accessory cells that include adipocytes and fibroblasts arranged on a delicate reticulin or collagenous network. This supporting stroma is not readily obvious until the hematopoietic tissue contracts in a centripetal manner with advancing age or is lost due to injury (Fig. 2).

Kinetically, the vast potential of this organ is revealed by its capacity for autologous reconstitution after severe but reversible damage. Subliminal whole-body radiation empties the marrow of its usual occupants, but after a variable period, they gradually return. Furthermore, segments of otherwise irreversible aplasia can be seeded from a shielded spleen or single limb, establishing the existence of a migratory pluripotential population. Such stem cells have the capacity to move from the circulation to a particular geographic area or niche, settle, and produce trilineage hematoopoiesis, suggesting that the apparently inert stroma has a rather more important role to play than previously anticipated.

Mature cells, such as reticulocytes, were shown to leave the marrow by amoeboid movement, whereas granulocytes became increasingly deformed so that they can enter the bloodstream through slits in the vascular endothelium. These phenomena were not initially appreciated, probably because of a preoccupation with the large numbers of relatively mature cells present under basal levels of hematoopoiesis or because of the dramatic expansion induced by stress. With technological advances, the previous concepts that blood forms in largely static terms needed to be modified to acknowledge the more dynamic nature of the process.

More attention has been focused on function since the demonstration, in short-term clonogenic assays, that mononuclear cells recovered from peripheral blood or marrow would form aggregates in response to a variety of stimulatory peptides. Elucidation of these regu-

![Image](image_url)

FIG 2. Organization of bone marrow. Left, bony trabeculae surrounded by easily recognizable megakaryocytes and other hematopoietic precursors. Right, the stroma can only be seen clearly after these megakaryocytes and other precursors are reduced. Lymphocytes, adipocytes, and plasma cells also can be identified. The function of the inductive microenvironment is to present stimulatory and inhibitory molecules to the stem or to the progenitors, either directly or through an associated matrix, thus regulating the entire sequence of blood formation, with the subsequent release of the mature elements into the circulation.

atory mechanisms centered on exposing the progenitors to growth factors, singly or in combination, and establishing correlations with the numbers of colonies formed and their composition.

Such a model was clearly rather simplistic, and experiments with two inbred mouse strains sharing a common phenotype of refractory macrophage anemia, an absence of mast cells, and changes in coat color showed that either a defective stem cell or a faulty stroma could be incriminated. These cellular facts established a central role for the latter, with its accessory cells that are in intimate contact with one another and are loosely grouped together to make up what is now described as the inductive microenvironment: the laboratory counterpart is the long-term marrow culture.

From a practical standpoint, much of this information can be brought together in a functional model. Here, a given level of homeostatic demand is selectively met by means of the appropriate ligand being presented by the stromal matrix and engaging its cognate receptor on a corresponding hematopoietic progenitor. Such a linkage initiates a signal that is transduced through a series of cytoplasmic substrates to the nucleus. The last molecule in this cascade is the
transcription factor, which has the capacity to activate genes that, in turn, generate their corresponding proteins or enzymes to determine a particular cellular activity. These range from entry into the cycle to the other extreme, known as apoptosis or programmed cell death.

Considered physiologically, it is possible to categorize the various forms of bone marrow failure. Initially, these forms can be divided into those having either a genetic or an acquired defect and, subsequently, they can be subgrouped according to whether one or more lineages are predominantly affected. This approach is attractive because it links the lessons learned from experimental hematologists directly to the clinic and establishes a rationale for current management strategies. To this end, it is illustrative to review, in a little more detail, what is known of some of these processes.

ANIMAL MODELS

The discovery of ionizing radiation dramatically influenced world events in the last half century. The potential for a nuclear holocaust has been offset, to some extent, by major benefits to the sick and suffering, including the application of ionizing radiation to the dissection of hematopoiesis.

Early work rested heavily on the use of this modality, leading to the development of new concepts such as the existence of renewal systems for many organs, including the bone marrow. The pattern whereby this physical force produced injury led to three cardinal observations. First, increasing cumulative doses eventually produced irreversible marrow damage, and recovery in those early studies masked simultaneous impairment of the stroma. Second, different target organs, exemplified by the central nervous system and the gut, had distinctive responses and, in the case of bone marrow, outcome could be modified by transplantation. Third, shielding of the spleen or a femur from lethal doses was followed by autologous reconstitution, establishing that stem cells could migrate, and this paved the way for modern sequential hemi-body radiotherapy regimens. These seminal observations from intact animals led to the introduction of clonogenic and long-term marrow-culture systems, with combinations of these techniques continuing to play a central role in unraveling hematopoiesis. Laboratory-based research has been linked with advances in medicine, spawning the industry of bone marrow transplantation—a major and often life-saving option when bone marrow function is irreversibly damaged.

THE HEMATOPOIETIC CELLS

The precursors of hematopoietic cells are morphologically well characterized, albeit heterogeneous, occupants of the marrow cavity. Of these, some retain the capacity to divide or proliferate. Most, however, undergo maturation in postmitotic pools; among these are reticulocytes, myelocytes, and later forms, as well as the distinctive megakaryocytes.

The progenitors are identifiable only by their ability to generate aggregates of progeny in culture systems. Such clonogenic assays require appropriate stimulating factors to be constantly released from feeder cells or to be present in the culture medium. Based on the growth factors provided, different cell types could be recognized. When these were a mixture of granulocytes and macrophages, they were designated GM-CFUc, an acronym for colony-forming units in culture.

The stem cells can similarly be defined only in functional terms. Totipotentiality resides exclusively in the zygote. Further growth leads to a series of tissue-specific families that are pluripotential, with one of these giving rise to the hematopoietic tissue. In irradiated rodent models, these cells form white masses in the spleen that contain all the normal lineages, and such colony-forming units are therefore designated CFUs. The same phenomenon can be repeated by homogenizing and retransplanting each of the aggregates, thereby demonstrating the repopulating potential of the earliest or ancestral cells.

It follows that, although they are few in number (accounting for only between 0.05% and 2% of the total nucleated marrow cells), CFUs have the intrinsic capacity to self renew and so give rise to the entire spectrum of the lymphomyeloid and probably even stromal lineage of each individual and to do so under basal as well as under stressed conditions. Such pluripotentiality is a vital resource that has naturally occurring protective mechanisms, exemplified by the majority of them resting in a quiescent phase of the cell cycle. Maintenance of arrest in this state can be achieved pharmacologically with agents like medroxyprogesterone acetate or macrophage inflammatory protein-1α, and these are used to provide myeloprotection during chemotherapy.

A second consideration is the intriguing conundrum of how these cells, which are predominantly sessile within the marrow extravascular compartment, migrate during fetal ontogeny from yolk sac to marrow and thereafter comprehensively maintain blood formation. Exploiting differences in physical properties, expression of cell-surface antigens, and cell-cycle status, it has become clear that there is considerable heterogeneity within this pool. Conceptually, there exist clones of varying maturity, with the most primitive constituting the reserve on which subsequent generations draw to replenish their numbers; the age-generation theory of stem cell descent. Such a continuum of immature hematopoietic ancestors ranges from pre-CFUs, with a capacity for long-term marrow repopulation, gradually merging into other bone committed compartments that have lost the abili...
ility to establish stable grafts. The marrow progenitors are migratory, and their low concentration in the circulation expands dramatically after aggressive therapy for malignant disease, at which time they can be readily collected and used for peripheral blood autografting.13

These stem cells have been designated long-term culture-initiating progenitors because of their in vitro behavior. However, it is not yet clear whether those stem cells derived from the peripheral blood, as a different anatomical site will prove identical in clinical practice when they are used for autologous transplantation.13

Another intriguing issue is how the earlier stem cells are enticed to leave their resting phases and contribute to renewal, differentiation, and, through lineage commitment, to proliferation, with consequent maturation. Is this a random or stochastic event, in contrast to its being determined by need? Available evidence suggests that the two processes for recruitment coexist in maintaining hematopoiesis, whether this is at the constitutive level or whether it occurs when the entire organ is induced to respond to stress. Furthermore, this applies throughout all phases of development.14 Uniform and modest overproduction of the marrow-repopulating cells constantly fill microenvironmental niches or undergo apoptosis because of lack of growth factor support.15 The homing of these cells to specific geographic sites probably reflects the expression of vascular cell adhesion molecules by the stroma, with subsequent growth and maturation by members of the macrophage lineage that produce the appropriate cytokines. Incremental engraftment into nonmyeloablated mice suggests that an exchange occurs between marrow and circulating cells.14

The transmembrane receptors that facilitate binding undergo constant change and, in this way, determine the localization of stem cells within the microenvironment. Thus downregulation of adhesion molecules assists the release of stem cells into the circulation. This type of interaction is believed to underlie the subtly different patterns of reconstitution that are based on competitive repopulation, in which the less mature marrow cells replace clones from the peripheral blood whose progeny have reached the end of their life span. In the context of chronic granulocytic leukemia, a not dissimilar hematopoietic competition may offer an explanation for graft-versus-leukemia effect.

One of the intriguing questions is “What do these cells look like?” Morphologically, they are relatively nondescript, resembling small lymphocytes. Immunophenotypically, the most distinctive antigen is CD34. Coexpressing this antigen with others, including CD33, human leukocyte antigen (HLA)-DR, or CD15, however, reveals that CD34 is already committed to the granulocytic–macrophage lineage. Similarly, irrespective of whether bone marrow, peripheral blood, or cord blood is studied, presence of the α3β1 transmembrane receptor with

tyrosine kinase activity is associated with erythropoiesis and granulopoiesis; its soluble form, released by normal human endothelium, may regulate the bioactivity of stem cell factor at the stromal level. This knowledge can be exploited by using immunologic techniques capable of harvesting the progenitors that can result in stable engraftment. At least in those derived from human umbilical blood, Thy–1 is expressed and derepresses inhibition of immature hematopoietic cells. Thus sources other than the marrow become attractive for autografting because they might, theoretically at least, have a lesser contamination with malignant cells. However, when those cells harvested from the peripheral circulation are considered for allogeneic transplantation, it becomes necessary to introduce some method for removal of the more abundant T lymphocytes, which, because of their larger numbers, regularly give rise to acute and chronic graft-versus-host disease (GVHD). A further word of caution is necessary. The use of peripheral blood mononuclear cells recovered after myelosuppressive chemotherapy for autografting, when peripheral blood mononuclear cells are defined only by the presence of the CD34 antigen, does not uniformly correlate with growth in clonogenic assays, and the latter may be a more reliable means for defining the repopulating potential.

Having arrived at this point, it is instructive to recall briefly those monumental studies in which the various cells, initially of marrow origin, were grown in culture and so focused attention on the whole growth factor field.

CLONOGENIC ASSAYS

Unraveling the organizational complexity of even a single tissue is a daunting challenge when isolated phenomena are considered, let alone when one is faced with understanding how blood formation is regulated in the intact organism. Without the capability to examine each sequential event in a culture system, it would not be possible to take the first step in identifying and characterizing growth factors. Only once this issue had been solved could attention be turned to the modulating influence of negative regulators. The logical extension of these studies was an exploration of the way in which receptors on stem and progenitor cells are brought into contact with their cognate ligands in the microenvironment.

The seminal observation was that growth could be induced in soft gel or agar using clones that contained mature leukocytes.15 16 The central requirement for growth was the presence of some factor in the medium, and because more than one clone of cells emerged, it appeared likely that there may exist a variety of peptides, each capable of stimulating the development of a different lineage. An important
Refinement was the use of serum-deprived conditions, in which exposure to pure growth factors showed that there was interplay among these, each sequentially activating specific genes that resulted in orderly differentiation. This technology continues to be applied to clarify the role of associated or permissive factors, including the interleukins (ILs). Once normal regulatory processes are understood, they become the basis for exploring the pathophysiology that underlies many hematopoietic disturbances. Furthermore, and of increasing importance, this knowledge has been extended to include the use of recombinant human molecules as potentially valuable therapeutic agents. However, there is already concern about the inappropriate use of recombinant human molecules; this is discussed later.

**CELL LINES**

At any one moment, marrow-culture systems yield populations at varying stages of differentiation, proliferation, and maturation. Such heterogeneity makes it difficult to study mechanisms operating at a particular point of development. This limitation can be overcome by using well-characterized cell lines in which specific phenomena can be defined under relatively homogenous conditions.

A good example is erythropoietin, in which the hormone is synthesized and secreted in human hepatoma systems that have proven helpful in unraveling molecular events. Comparable approaches have made it possible to understand the subcellular actions of stimulatory peptides in which, for example, it has been shown that IL-11 acts as an autocrine growth factor for megakaryocytopenesis.

Of note is the availability of long-lived stroma that provide tools to isolate those regulatory influences exerted by the microenvironment, including the extracellular matrix and cell-adhesion molecules. Similarly, albeit in a mouse model, a nonleukemic counterpart has been developed that is suitable for investigation of molecular changes early in hematopoiesis at a level at which genes are employed in self-renewal. The applicability of this technology to intact animals has been established by parallel studies in freshly harvested tissue. This has led to the recognition that progenitors have a multilineage phenotype, which provides a plausible explanation for the phenomenon of lineage infidelity ascribed to genetic misprogramming resulting from molecular aberrations that follow chromosomal translocations.

**REGULATORY GROWTH FACTORS**

Stimulatory peptides were the first molecules to attract attention. This came from the demonstration that colony formation in culture systems could be enhanced by substances secreted into the medium from a variety of leukocytes and the recognition that these exert important physiologic control of hematopoiesis. Stimulatory peptides were soon seen to act either directly through large glycoprotein receptors expressed on the surface of progenitors or as response modulators, in which case an accessory cell in the hematopoietic microenvironment was stimulated and this, in turn, produced a growth factor, as in the case of IL-1. As the number of stimulatory peptides has increased and individual actions have been clarified, the peptides have been shown to fall into two categories. Some act broadly, such as GM-CSF, IL-3, and the c-kit ligand, which expand the committed progenitor population but facilitate proliferation of more than one cell type. Others are lineage specific, exemplified by erythropoietin, G-CSF, M-CSF, and by IL-5, all of which control late-stage maturation changes. In most instances, the two classes act together, bringing about optimum marrow output.

This rather simplistic approach has already required modification because the concept of a mobile ligand linking to its stationary receptor is not strictly accurate. The mobile ligand, instead of necessarily being free, may be membrane associated, whereas the stationary receptor has soluble isoforms. Distinction between the older key-and-lock analogy thus becomes blurred, and the regulation of many cell functions is seen to be much more dynamic, with variations suggesting alternative mechanisms for intercellular communication. However, purely in the interests of clarity, the more traditional understanding has been retained in discussing some of the clinically important factors.

Erythropoietin levels in the plasma increase exponentially with lowered tissue oxygenation, irrespective of its cause. The origin of the hormone is uncertain, but restriction of the gene to subsets of cells within the kidney and liver is consistent with the oxygen-sensing mechanism residing in one of these organs. Support for this contention is found in experimental studies using hepatoma cell lines or appropriate animal models, in which messenger RNA is generated in response to hypoxia or exposure to cobalt. On release, the protein binds to its cognate receptor, which is a member of the hemopoietin superfamily, whose common feature is a lack of intrinsic tyrosine kinase domains. A soluble isoform is now known to exist, although its physiologic importance awaits clarification.

Ligand engagement initiates a series of events that culminate in the production of erythrocytes. The delivery of an adequate signal to this differentiation pathway also requires activation of globin genes regulated through the common transcription factor, designated GATA-1. Next, with the cooperation of early-acting cytokines, including IL-3 or the c-kit ligand, there emerge primitive progenitors, designated burst-forming unit, erythroid (BFU-E). These progress to the more ma-
ture CFU-E, which are extremely sensitive to low concentrations of erythropoietin. Maturation through recognizable normoblasts to erythrocytes is critically hormone dependent and usually occupies 10 to 20 divisions, with a prolonged S phase, but this stops at the level of orthochromatic cells that are arrested in G0.

Once erythropoietin was purified, it could be used to unravel interactions with other growth factors that affect this lineage. Predictably, erythropoietin was transferred to the therapeutic arena, in which its use has been beneficial in many diverse conditions, including anemia of chronic renal failure and myeloma.

Granulocyte-stimulating factor (GSF) is the prototype of late-acting molecules originally derived from the 5637 bladder cancer cell line, and its production is controlled by a gene located on the long arm of chromosome 17. The closely related macrophage and granulocytemacrophage peptides, the latter generated from a site on the long arm of chromosome 5 in close proximity to other glycoproteins that form part of a superfamly, share an ability to influence immunohematopoietic cell proliferation and function. 20

Cytokines, as with the interleukins, have highly conserved and overlapping amino acid sequences, which are essential for binding to their membrane-associated receptors. Such shared subunits help to explain both redundant and pleiotropic functions (Fig. 3). 20 In general terms, ligand attachment causes conformational changes in the transmembrane glycoprotein, with resultant modification of the intracytoplasmic domain. For example, there is induction in protein tyrosine phosphorylation or alteration in phosphatase activities. 23 Such steps initiate the transmission of signals to the nucleus that will, in turn, activate genes to bring about the desired alteration in cell function.

The availability of growth factors has contributed greatly to elucidating subcellular phenomena. In parallel, growth factors have been used for successfully treating many patients with bone marrow failure but, regrettably, commercial expediency has, in many cases, overtaken responsible use. 22 Certain oncogenes, exemplified by c-myc and c-fos, are activated as early events when stimulated by these products, and when taken in conjunction with their other observations, introduce a note of caution about potential adverse effects, particularly when these stimulatory peptides are used in high concentration. 23

The interleukins are a group of peptides that mediate communication, primarily among white cells, although their activities extend to nonhematopoietic tissues (Table 1). 24 The interleukins share many functions with the traditional growth factors, including common receptor binding and coagulation with them in the development of a variety of cell lines. In addition, the interleukins participate in the

**TABLE 1. The interleukins**

<table>
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<tr>
<th>Interleukin*</th>
<th>Primary cell of origin</th>
<th>Major function</th>
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<tbody>
<tr>
<td>1</td>
<td>Macrophage</td>
<td>Host defense</td>
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<tr>
<td>2</td>
<td>Lymphocytes</td>
<td>Antitumor</td>
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<tr>
<td>3</td>
<td>Activated T cells</td>
<td>Autoimmune</td>
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<td>CD4+ T lymphocytes</td>
<td>Longevity</td>
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<td>5</td>
<td>Activated T cells</td>
<td>Eosinophil</td>
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<tr>
<td>6</td>
<td>Activated T cells</td>
<td>Stimulation</td>
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<tr>
<td>7</td>
<td>CD4+ T lymphocytes</td>
<td>Eosinophil</td>
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<td>8</td>
<td>Phagocytes and</td>
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<td>9</td>
<td>Activated T cells</td>
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<tr>
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<td>CD4+ T lymphocytes</td>
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<td>13</td>
<td>Mononuclear cells</td>
<td>Stimulation</td>
</tr>
</tbody>
</table>

*Many of these molecules act synergistically with growth factors and share with them redundancy, reflecting their production by many different cell types, and pleiotropy, acting on more than one target and doing so within the hematopoietic, lymphopoietic, inflammatory, and nervous systems.

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acute-phase response to inflammation. In a like manner, they often act on early progenitors and cooperate with similar cytokines, such as the c-kit ligand, to facilitate production of both red and white cells as well as platelets.22 Conversely, the effects of interleukins may be exerted somewhat later, that is, after lineage commitment, as seen with monocytes and granulocytes or when ILs 6 and 11 affect, primarily, thrombopoiesis.19 As these molecules have been isolated, sequenced, and cloned, pure recombinant proteins have become available. This has been helpful in the exploration of both the complexities of hematopoiesis and the way in which this physiologic process overlaps with inflammation and immunologically mediated events. Predictably, these biologic products have been introduced into the clinic, and their therapeutic potential has already been recognized. Nevertheless, it is again essential to emphasize that prudence and scientific restraint are needed to prevent the irresponsible overuse of these pure recombinant proteins. Their use must be neither exploited commercially nor discredited through enthusiastic but inappropriate patient treatment.22

Transmembrane receptors that initiate signal transduction are made up of alpha, beta, and in some cases, gamma, subunits that, in various combinations, may be shared by the respective growth factors, and it is this phenomenon that contributes to both redundancy and pleiotropy.23,25 Additionally, these structures have similar free transmembrane and intracytoplasmic domains, with the latter participating in two major phosphorylation and dephosphorylation reactions as the first step in the onward passage of information to the nucleus.26

The enzymes catalyzing these processes in eukaryotes are protein kinases, with their complementary phosphatases. In the first of these processes, the phosphate is transferred to serine or threonine residues, in what is known as the G-protein-coupled pathway. Here, receptor binding leads to activation of protein kinase C by diacylglycerol in a calcium-dependent step.27 The intermediary stages in the process of cell-function modification are uncertain, but it is possible that the enzyme itself may directly bind to nuclear proteins or substrates that include the topoisomerases. The second is the tyrosine kinase pathway that involves many cytoplasmic proteins or enzymes, with at least some of these sequentially interacting with ras and raf protooncogenes and the MAP-kinases.3,27

In both examples, transcription factors bind to specific DNA segments, including oncogenes such as c-fos and c-myc, through a particular conformation or structure, exemplified by the zinc-finger motif. These may, in consequence, activate or repress programs for cell-cycle progression, replication, or apoptosis.28,29 Of relevance is the recent demonstration that short segments of nucleic acid can be synthesized to have subtly different structures from those of their naturally occurring counterpart. These antisense oligonucleotides can bind or hybridize to the normal cell product according to the rules of the genetic code and so may prevent its translation into the corresponding protein or enzyme. This technology may be applicable for use in switching off a critical intracellular event and may also prove useful to define regulatory mechanisms experimentally.

Permissive factors have little intrinsic activity but potentiate early hematopoietic events and thus generally affect multiple lineages. Examples of this include the c-kit ligand, basic fibroblast growth factor, and IL-6.28 Understanding these activities was facilitated by the use of a mouse model in which a common phenotype could arise in two different ways. Mutation at the white spotting (W) or Steel (S) loci affected, respectively, generation of the transmembrane protein kinase receptor c-kit or production of its cognate ligand.

An analogous situation occurs in humans. Here, the stem cells expressing the CD34 antigen are brought into contact with the Steel factor, which is synthesized by stromal cells, in part because of the stromal cells providing adhesion sites between the progenitor and environmental matrix. Once the c-kit protooncogene is activated, conformational changes take place in the ectodomain, and dimerization follows, after which autophosphorylation of the tyrosine kinase in the cytoplasmic segment leads to signal transduction and appropriate lineage expansion. These early events are well exemplified in the case of erythropoiesis, in which progenitors are amplified up to this stage of globin production, but then interaction with erythropoietin is necessary to complete their maturation (Fig. 4).

Similarly, basic fibroblast growth factor has limited intrinsic clonalogenic activity, but it acts synergistically with stem cell factor in narrow niches.40 Culture studies suggest that in its biologic form, a complex is generated with proteoglycans from the matrix, and only then does basic fibroblast growth factor modulate megakaryocyteopoiesis and other hematopoietic activities. For example, based on studies using a human erythroblastic leukemia cell line K562, basic fibroblast growth factor increases the pool of progenitor cells by antagonizing the effect of those cytokines that would otherwise induce their differentiation.

Other substances influencing blood production are the somatomedins, heme, hepatocyte growth factor, the leukotrienes, and perhaps not surprisingly, an influence on hematopoiesis for the sympathoadrenergic system. Although a number of these interactions remain controversial, preliminary results illustrate the extent to which our concepts must constantly be modified to keep abreast of new advances. Nowhere is this more true than in the need to develop a sound understanding of regulatory mechanisms so that these very powerful molecules can be responsibly used in therapy, such as in patients with bone marrow failure, albeit at a tangent to the issue.
Increasingly, attention is turning to the role that these molecules play in clinical practice. This is illustrated by the upregulation of gamma interferon-induced suppression of bone marrow progenitor cells in the pathogenesis of aplasia, although it is interesting that such effects may be overridden by growth factor combinations. These products are entering the therapeutic arena; they can be administered to protect stem cells from damage during treatment with alkylating or antimetabolite agents, including, as we have seen, medroxyprogesterone acetate or macrophage inflammatory protein 1-α.

THE HEMATOPOIETIC-INDUCTION MICROENVIRONMENT

The overall organization of the hematopoietic-inductive microenvironment is both anatomically and functionally geared to interact with pluripotent stem cells and provide geographic areas where these cells can reside and grow. The cellular components are heterogeneous and include adipocytes, members of the immune and lymphoreticular systems, fibroblasts, macrophages that lie free but may be found at the centers of erythroblastic or plasmacytic islands, and reticular and vascular endothelium. How these different populations contribute to hematopoiesis was impossible to determine until segments could be isolated in the laboratory using long-term marrow cultures. In spite of the availability of this technology, many questions remain unanswered. One such question is whether a corresponding role exists for the spleen in hematopoiesis. Another is the emerging concept that there may well be a common stem cell for both marrow and lymphomyeloid lineages. Additionally, there is an increasing appreciation of the need to distinguish between physiologic and pathologic responses in the hematopoietic microenvironment in hematologic disease and treatment. Lymphocytes are prominent, and those of T lineage, whether natural or lymphokine-activated killer cells, can experimentally exert both stimulatory and inhibitory effects on the stem cells. The mechanisms of action appear to be primarily through the release of soluble factors, some of which affect the early phases of differentiation and proliferation, with subsequent modification by more lineage-specific molecules that regulate maturation and cell release. To an extent, these interactions are bidirectional, with cells of the immune system also playing a role in the reconstitution that follows transplantation. Similarly, marrow failure appears, in certain circumstances, to result from enhanced expression of negative regulators, and a correlation exists between some of these clinical entities and quantitative or qualitative disturbances in the CD4+ population.

With this background, it was predictable that attempts would be made to exploit therapeutically the function of these accessory cells.
To date, examples include stimulation by bryostatin-1 that upgrades the in vivo production of IL-3 or GM-CSF by T cells. In a similar context, the impaired cytokine production that occurs with increasing age has been used as a model to gain insight into the regulation of genes that encode stimulatory molecules such as GM-CSF and then to develop strategies for modifying hematopoietic response in the elderly.

The vascular endothelium was for many years thought of as a simple mechanical barrier between blood and marrow. However, this is a dynamic population, with functions that include the synthesis of naturally occurring anticoagulants and regulation of cellular or molecular traffic by transendothelial or interendothelial routes. Furthermore, the vascular endothelium constitutively produces a range of stimulatory peptides that modulate myelopoiesis and can be induced by oncostatin M to generate G-CSF and GM-CSF. This information collectively underlines the important role that these intimal cells play in the physiologic regulation of granulopoiesis.

Insight into the anatomic relationships in the stroma comes from the study of long-term cultures, in which a blanket cell forms an epithelial layer that separates proliferating hematopoietic cells from the supernatant, with the former regarded as the in vitro equivalent of the extravascular compartment. These investigations have been aided by using cellulose acetate membranes, on which microvilli meet and trap migrating cells, thereby facilitating their perforation of the epithelial layer to gain entry into the cellular compartment. More recently, refinement has been added by introducing a microporous membrane between the hematopoietic progenitors and the stroma. These studies have demonstrated that direct contact between the two populations is not necessary for conservation of primitive hematopoietic cells, but it is essential for the regulated production of mature blood elements. This occurs through stroma-derived factors, which are probably different from those of the early-acting cytokines.

Adhesion molecules are centrally involved in guiding the movement of cells within the microenvironment, either by providing attachment to the stroma or by the facilitating release of maturing precursors. These are often grouped together in families. Examples are the cadherins, which bring about cell-to-cell binding through calcium-dependent mechanisms called the integrins, which are the prime mediators for attachment to the matrix, with protein being their major structural component; the selectins, in which this role is played primarily by carbohydrates; and the immunoglobulin superfamily, which is concerned with antigen recognition and adhesion, particularly in the T lymphocytes.

Many of the stromal regulatory events require reciprocal interaction between accessory cells in the bone marrow, including those of the vascular endothelium and hematopoietic stem cells. It is notable that such relationships appear to center on interdigitating microprocesses on the accessory cells in the bone marrow but are also found on a wide variety of other cells, including those that express the CD4 antigen and others of immunologic importance. Again, the parallels between such subcellular events and their clinical application can be enlisted to explain a number of phenomena. For example, this might underlie the retention of blasts in the marrow or may govern the localization of lymphoma cells in bone marrow through the linking of integrin to its ligand in the stroma, with upregulation of either molecule leading to increased adherence.

The extracellular matrix provides the final meeting ground for this wide range of cells, including their receptors and the corresponding ligands that are synthesized by the stroma. The extracellular matrix is a structurally complex tissue comprising a variety of collagens, laminin, fibronectin, and proteoglycans, all of which combine to provide physical support for the progenitors and dynamically orchestrate their orderly development. Of interest is basic fibroblast growth factor, which forms a complex with heparan sulfate, and in this specific environment brings together those cytokines that control, for example, human megakaryocyteopoiesis. The importance of these extracellular proteins was further demonstrated using a plastic immobilization model that linked thrombopoietin to the c-kit ligand and generated a signal leading to colony formation.

In a way, these experiments have come full circle, with niches that originally had largely morphologic connotation shown to be the dynamic interface between the progenitor and the inductive microenvironment. Direct applicability to bone marrow failure and its management is found in competitive repopulation experiments in which conventional wisdom is contraindicated by demonstrating that long-term engraftment can take place without prior conditioning, which was previously advocated as being necessary to make space for the incoming graft. Confirmation of this phenomenon in human studies would establish a precedent for infusion without first conditioning the recipient by chemoradiotherapy and would strengthen the argument for the use of stem cells as vehicles in gene therapy.

LONG-TERM BONE MARROW CULTURES

Clonogenic assays that were first used to provide information about stem cell response to positive and negative regulators were limited in that they reflected only a small segment of the hematopoietic process. Little insight could be obtained about the vastly more complex situation that governed homeostasis of blood formation in the intact animal, particularly the role of the inductive microenvironment, with its many accessory cells and molecular matrix. The seminal step in
overcoming this limitation was a demonstration by Dexter et al. of a method in which adherent stromal layers were established and then inoculated with bone marrow cells. The Dexter method was then refined and applied to humans, although some controversial issues persist. For example, there is debate about whether a single progenitor gives rise to stroma and stem cells or whether such commonality might be explained by differences in the technology used. Similarly, the importance of natural killer cells has also been examined, and it is noteworthy that these and possibly other lymphoid lineages are generated directly from progenitors before there being any phenotypic evidence of lymphoid or myeloid commitment. Nevertheless, this model deserves credit for the improved characterization of hematopoiesis, particularly the crucial regulatory interactions among stem cells, cytokines, and the inductive microenvironment.

Dexter's original method has been further modified to look at blast cell adherence. Here, the earliest phenotypes are presumed to express receptors for adhesion molecules whose ligands are generated by the stroma and so lead to their retention within the niches. Indeed, some of the early-acting molecules, exemplified by both Steel and basic fibroblast growth factor, appear to function in this way, either singly or very possibly in collaboration. Subtle alterations in this type of binding would allow the slightly more mature progenitors to pass through the agar into the supernatant, where they can be characterized in standard clonogenic assays based on their response to appropriate cytokines.

The capacity independently to manipulate these two major populations (stroma and hematopoietic stem or progenitor cells) has clear advantages and resulted in, among other things, characterization of the ancestors that can comprehensively regenerate lymphohematopoiesis and undergo in vitro expansion. In human studies, this is described as the high proliferative potential colony-forming cell and can be recovered from the peripheral blood in sufficient numbers for autografting. Although present in very low concentration, these are amenable to growth in suspension cultures, stimulated by a variety of early-acting cytokines such as IL-1 and Steel ligand, but this can also be achieved using molecules that are active when used on their own. It has emerged that primitive hematopoiesis can be initiated without stem cell factor.

The Dexter system and its blast colony modification paved the way for the better understanding of the physiologic mechanisms regulating hematopoiesis by accessory cells of the stroma and associated matrix. As the characteristics of the earliest progenitors were defined, including in particular their capacity to initiate and sustain long-term clonogenic engraftment, techniques have emerged to refine autografting by selectively enhancing the concentration of such recombining units and quantifying the units. Furthermore, corresponding fractions can be obtained from umbilical cord blood or from the peripheral circulation, the latter particularly after mobilization with recombinant human growth factors. Whether corresponding fractions are taken from umbilical cord blood or from peripheral circulation, it is possible to expand the very early committed lineages in vitro using a variety of growth factors. After their infusion, blood counts recover rapidly, facilitating early discharge of the patients from the hospital. It is notable that these maneuvers are swiftly being adapted to clinical practice, in which they have an established role in myeloprotection of individuals receiving escalating doses of chemotherapy. Their use can also shorten markedly the pancytopenic phase that follows bone marrow transplantation.

SUMMARY

It may appear that an inordinate amount of time has been devoted to describing the physiologic principles currently thought to underlie the regulation of hematopoiesis. This exercise is appropriate for three reasons. First, it bridges the gap from the early days of largely descriptive reports through an era in which experimental hematologists used radiation biology, cytogenetics, and marrow-culture systems to begin understanding the controlling mechanisms for blood formation. Second, the proposed model, which unifies many of these events, takes into account the surprising degree of heterogeneity in the stem and progenitor cell compartments, with their diverse response to stimulatory peptides or inhibitors, and emphasizes the cardinal regulatory role vested in the stroma. These various interactions can be used to classify rationally the syndromes of bone marrow failure. Third, with the more ready availability of recombinant human cytokines and an ever-widening choice of techniques for transplantation, some of the previously empiric approaches are being placed on a better footing. Examples include attempts to modulate hematopoiesis immunologically at the microenvironmental level through the use of antilymphocyte globulin, bryostatin-1, and immunosuppressive agents, ranging from corticosteroids to cyclosporins. In the therapeutic context, however, it would be less than responsible if concerns about inappropriate use of these valuable products were not emphasized.

OUTLINES OF PATHOPHYSIOLOGY

An inherited as opposed to acquired defect is a distinction all too frequently forgotten. There exist a number of genetically determined lesions that are experiments of nature. These are golden opportunities to understand pathogenesis, and the use of modern technol
TABLE 2. An approach to bone marrow failure

<table>
<thead>
<tr>
<th>Congenital syndromes</th>
<th>Acquired defects</th>
</tr>
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<tbody>
<tr>
<td>Persistent single lineage</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Josephs–Diamond–Blackfan anemia, congenital dyserythropoiesis</td>
</tr>
<tr>
<td></td>
<td>Chronic idiopathic neutropenia, cyclic neutropenia, Kostmann’s syndrome, Myelodysplasia</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia with absent radii</td>
</tr>
<tr>
<td>Platelets Evolving to marrow aplasia</td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Fanconi’s anemia, Dyskeratosis congenita</td>
</tr>
<tr>
<td></td>
<td>Shwachman’s syndrome, Reticular dysgenesis</td>
</tr>
<tr>
<td>White cells</td>
<td></td>
</tr>
</tbody>
</table>

The great diversity of these syndromes can be simplified by first dividing them into inherited or acquired categories. Inherited syndromes are generally irreversible, whereas those with acquired syndromes may recover spontaneously. Within each of these two broad groups, single-lineage defects may occur and remain unchanged. In contrast, other patients may have anaemia, neutropenia, or thrombocytopenia that evokes relentlessly to global failure or aplasia.

can then provide new information about the way in which lymphohematopoiesis is regulated (Table 2).

Spontaneous recovery is a major consideration that should be contrasted with irreversibility. Thus in many clinical situations, as well as infections or drug-related damage, the pancytopenia or hypoplasia will turn out to be self-limiting. Of more ominous significance are those cases in which relentless loss of hematopoietic tissue occurs. This is seen typically in congenital disorders or when the extent of the damage is so great that spontaneous recovery is precluded. In the last category are the effects of industrial toxins such as benzene or irradiation, in which cumulative effects erode the stem cell compartment beyond its capacity for regeneration.

The natural history of bone marrow failure is variable. Many cases start by involving a single lineage, for example, anaemia, leucopenia, and thrombocytopenia, and these may remain life-long. Isolated phenomena. In other patients, there is the same initial peripheral blood finding, but it evolves to pancytopenia with aplasia. Here there is a much greater management problem and, although recombinant growth factors are useful, autologous bone marrow transplantation currently offers the only realistic chance for cure. This procedure is relatively crude so that there is an overwhelming need to identify those individuals in whom there is a single gene at fault and in whom selective replacement is an ever-increasing reality.

CONCLUSIONS

These syndromes are all too commonly encountered in clinical practice, with outcome being determined by early recognition, accurate diagnosis, and appropriate therapy. These patients are seen by hematologists, specialists in internal medicine, and generalists alike, so that it can reasonably be argued that an understanding of hematopoiesis is necessary for all medical practitioners if optimal care is to be provided. To this end, recent advances in cellular and molecular hematology can be brought together in a progenitor cell model, and this should now be regarded as part of our general knowledge.

Moves are already afoot to refocus the effective approach of hematopoietic stem cell transplantation along much more sharply defined avenues in which defective genes, once identified, can be selectively replaced. Finally, if that is the bright side of our future direction, there is also a sobering perspective. Sadly, this is to be found in the epidemic of acquired immunodeficiency disease, which continues to sweep across the globe. Infection with this retrovirus has forever changed the face of lymphoproliferative disorders and is in the process of creating a whole new category of defects in hematopoiesis— including bone marrow failure.

CONGENITAL SYNDROMES: PERSISTENT SINGLE LINEAGE INTRODUCTION

In early life, anaemia, neutropenia, or thrombocytopenia can occur in isolation and fail to remit spontaneously. In some instances, these inherited defects remain restricted to a single cell line, as in the Josephs–Diamond–Blackfan anaemia and amegakaryocytic thrombocytopenia with absent radii. Conversely, the Shwachman–Diamond syndrome and reticulocytopenia may progress to pancytopenia as a consequence of trilineage marrow aplasia.

These are all examples, to a greater or lesser extent, of bone marrow failure, in which the causative lesion is seldom elucidated. There is, however, some evidence to support the existence of genes that, by their expression or repression, predispose to the different entities. In this context, it is reasonable to postulate that homozygosity would favor global hematopoietic involvement, as described by Fanconi. In contrast, heterozygosity may underlie fractures, in which solitary cytopenia can evolve into more widespread damage. Furthermore, it is conceivable that such a genetic deficiency creates an unstable chromosomal complement and, thus, predisposed to relatively minor environmental insult may precipitate the overt hematologic lesion—a point astutely predicted more than a half century ago. It follows that the various syndromes could be considered to be part of...
a common spectrum on the basis of the proposed progenitor cell model for hematopoiesis. The separate description of each syndrome is somewhat artificial but is a pragmatic solution; accordingly, for each syndrome, the clinical and hematologic defects are described, and the underlying pathophysiological mechanisms are used as a basis for summarizing an approach to therapy.

ERYTHROCYTES

Josephs–Diamond–Blackfan Anemia

This congenital erythroid hypoplasia occurs in families and is associated with a variety of extramedullary abnormalities. This disorder was initially reported to be normochromic and normocytic; however, in most cases, the mean cell volume is raised, but vitamin B₁₂ and folate levels remain normal.⁴⁶ Leukocyte and platelet counts are typically unaffected but may be reduced in some patients.

Historically, constitutional anemias were recognized as early as 1911, but it remained for Josephs, in 1936, to describe two infants who had an isolated failure of erythropoiesis. In a larger review of 175 cases, the congenital nature of constitutional anemia was confirmed, with 12% of the diagnoses being made within 2 months of birth and 72% of the diagnoses made by the age of 4 months, with abnormal thumbs, webbed neck, and growth retardation often occurring in these infants.⁴⁶ In 1951, the landmark observation that this disorder was responsive to therapy with steroids was reported. Currently, however, the use of recombinant human growth factors or bone marrow transplantation is preferred.

Clinically, the diagnosis becomes evident when progressive anemia with skeletal abnormalities is found in the newborn. Although not all the defects are necessarily present in one individual, the infant may have high-arched palate, inguinal hernia, absent ribs, growth retardation, horseshoe kidney, and cardiac septal defects, with radiologic changes usually limited to the thumb or cervical vertebrae.

The natural history of Josephs–Diamond–Blackfan anemia has changed little from the first description, with spontaneous remissions being infrequent. Treatment by splenectomy was not effective, and although red cell transfusions helped to prolong life, these transfusions led to siderosis. Outcome was significantly improved by the use of corticosteroids, which not only arrested growth retardation but also resulted in remission in some cases. Although it is unusual, the diagnosis of Josephs–Diamond–Blackfan anemia may first be made in adulthood. Long survival may be associated with neoplasia, such as hepatocellular carcinoma.

Hematologically, mean red cell volumes are generally above 100 fl. In addition, serum erythropoietin is present in the face of raised erythropoietin levels, and persistence of fetal hemoglobin or expression of the f antigen is frequently found. Leukocytes and platelets are typically unaffected, although neutropenia, thrombocytopenia, and thrombocytopenia can occur. Bone marrow examination shows isolated erythroid hypoplasia, which is confirmed by ferrokinetic studies.

Immunologically, a number of lesions are found, with blood lymphocytes inhibiting red cell production by marrow progenitors from normal controls⁴⁷ and autologous reconstitution after immunosuppression or the use of prednisone. One interpretation of this is that the operation of an immune mechanism renders erythroid precursors insensitive to erythropoietin, particularly when a crude hormone is used.⁴⁸ Other growth factors, however, may also be involved, whereas occasional extension to leucocyte and platelet lineages raises the question about whether an earlier or less committed progenitor might be the target for this genetic lesion.

Biochemically, red cell enzyme assays can be coupled with the hematologic findings to separate congenital hyperplastic anemia from related conditions, particularly transient erythroblastopenia of childhood.⁴⁹ In congenital hyperplastic anemia, the immune dysfunction, with its propensity to evolve into leukemia, has elevated levels of erythrocyte adenosine deaminase activity. This disturbance in purine metabolism has been advocated as a marker for identifying these patients, although similar levels may occur in Fanconi, hemolytic, and acquired aplastic anemia.⁵⁰ Changes in orotidine decarboxylase are less clearly understood with regard to the pathogenesis of congenital hyperplastic anemia.

In patients with Josephs–Diamond–Blackfan anemia, chromosomal analysis shows an increased number of random breaks, with abnormalities on chromosome number one, whereas pentasomy 21 has been reported to predict the subsequent development of acute leukemia.⁵¹

Clonogenic assays of these patients show that the T lymphocytes are normal, whereas in coculture studies, peripheral blood erythroid progenitors are deficient in either number or function.⁵² These observations contrast with a study that demonstrated persistence of lymphocyte-mediated suppression of BFU generation in two adults. Hematologic remission was then explained by postulating the development of a serum factor that blocked the suppressive effect on the red cell precursors by a population of autologous T cells. Additional support for immunologic mechanisms is found in lymphocyte dysfunction, reported in five patients. However, in only one of these was there a suppressive effect on erythropoiesis.

Cell lines have been studied relatively infrequently in these patients, although clinical similarities have been described with the W and S1 mutant mice. Tests on the proliferation of M-07 show evidence
that stem cell factor is produced constitutively by the microenvironment of patients with this congenital hypoplastic anemia, but the low number of receptors reported requires confirmation because, at least in two patients, both c-kit and its ligand were intact.53 The pathogenesis of this congenital hypoplastic anemia remains controversial. In early erythroid progenitors, aberrant colony formation is corrected after the addition of IL-354; this correction is most striking when stem cell growth factor and erythropoietin are added. It appears that this lesion worsens with time and, notably, is not restricted to the red cell line, which may explain the hematopoietic alterations associated with the increasing age of these children.50 The observation that three patterns of response are seen for the cytokine combinations supports the postulate that there may be more than one underlying molecular defect.56 There is speculation that this disorder arises from the genes coding for c-kit or its ligand, but failure to demonstrate abnormalities in either suggests that the primary pathology in most or all cases lies elsewhere57; indeed, erythroid failure has recently been characterized by accelerated apoptosis.58 Possibilities include impaired signal transduction or interference with transforming factors for gene activation. However, there is no support for a postulated defect in the stroma or for inactivation of antibody-mediated mechanisms, in spite of some tantalizing evidence of an underlying immunologic disturbance. Some researchers believe that these lesions arise independent of aberrant immunoglobulin production.

Diagnosis may be difficult in the first few weeks of life, when an infant has only anemia, for which alternative causes include blood loss, infection, immunohematology, aplasia, myeloma, and transient erythroblastemia of childhood.59 Notwithstanding this, a high index of clinical suspicion will generally lead to the examination of peripheral blood and marrow, with confirmation derived from clonogenic assays and cytogenetic studies.

Complications occur when multiple blood transfusions are given, leading to immunomodulation and compromising subsequent marrow grafting. Additionally, iron overload, culminating in hemochromatosis, is a potential hazard, and the use of high-dose deferoxamine, albeit costly, is a sensible option until definitive treatment is initiated.

Management with standard or high-dose corticosteroids is not without risk of endocrine and musculoskeletal damage or infections, whereas the addition of cyclosporin is largely anecdotal. The current therapy is based on the results of laboratory data and clinical trials and consists of recombinant human cytokines, particularly IL-3,58 in combination with stem cell growth factor.50 A significant number of patients respond to this therapy, whereas the role of other stimulatory peptides, such as GM-CSF and the addition of erythropoietin, awaits clarification. Ultimately, however, bone marrow transplantation seems to be the best option for these patients because of its curative potential.60 Here, the exposure of the graft to Campath 1G ex vivo, with its low rejection rate and abrogation of acute and chronic GVHD, is particularly attractive.60 It is not clear whether these results can be extrapolated to minimally histoincompatible family members or even to matched unrelated donors, in which much higher complication rates remain a problem.

**Congenital Dyserythropoiesis**

Congenital dyserythropoiesis embraces a group of hereditary refractory anemias unified by the presence of ineffective hematopoiesis. Distorted erythroblast morphology is typical; however, it is not specific, and white cell and platelet counts are unaffected. Usually, there is an increase in indirect bilirubin levels in the serum and an absence of haptoglobins, but the reticulocyte count is low for the hemoglobin level, in spite of marrow hypercellularity.

There are four subtypes of congenital dyserythropoiesis, which are separated by the degree of megakaryoblastic maturation, giant erythroblasts, dyserythropoietic morphology, serologic aberrations in red cell response to acidified serum or expression, and lysis by anti-I or anti-i (Table 3).60 The basic genetic defect of erythroid membranes occurs only in type II congenital dyserythropoiesis. In type II, these membranes lack the enzyme N-acetyl glucosaminyl transferase, which is concerned with glycosylation.63 This gives rise to increased susceptibility to lysis in an acidified normal serum, which may be related to a unique antigen that reacts with immunoglobulin M (IgM) antibodies that are found in a proportion of unaffected subjects. It is notable that this disorder may coexist with other genetic abnormalities, necessitating patients to more severe hemolytic. Further.

<table>
<thead>
<tr>
<th>Type</th>
<th>Inheritance</th>
<th>Familial occurrence</th>
<th>Ham's Test</th>
<th>Anti-I agglutination</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Recessive</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Macrocytic</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>Megablastoid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Erythroblast intercellular bridging</td>
</tr>
<tr>
<td>II</td>
<td>Recessive</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>Mildly macrocytic</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>Megablastoid</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Erythroblast intercellular bridging</td>
</tr>
<tr>
<td>III</td>
<td>Dominant</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>Multinucleated giant erythroblast</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Absent double erythroblast</td>
</tr>
<tr>
<td>IV</td>
<td>Uncertain or not clearly established</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Absent double erythroblast</td>
</tr>
</tbody>
</table>

*In addition to the four established subtypes, new variants are described regularly.
more, individuals who have received multiple blood transfusions acquire isoantibodies, causing the direct antiglobulin test to become positive and confusing the diagnosis.

The classification of congenital dyserythropoietic anemia is constantly being revised with the description of new variants, including the occurrence of primary shunt hyperbilirubinemia. Historically, these are relatively rare disorders, with the bizarre nucleated red cells sometimes referred to as giantstoblasts. The first descriptions appear to have been made in 1943, with the literature collated and reviewed by Berman in 1947. The familial nature of these disorders was recognized in 1951 by Heimjel and Wendt. Impetus was given to developing the currently favored classification more than 20 years ago. Details of the classification system were provided by ultrastructural studies, with further information provided by a number of investigators.

Clinically, most patients have this form of congenital bone marrow failure early in life, having refractory anemia, ineffective erythropoiesis, and gross distortion of erythroblast morphology. Recognition of the disorder may be delayed into adulthood, however. The presence of hemosiderosis and hepatic cirrhosis sets the stage for evolution to hemochromatosis, and bone marrow expansion gives rise to extramedullary hematopoiesis, including paravertebral masses. On occasions there may be associated hydrops fetalis in which, paradoxically, the mother has mild disease, but the more serious variant occurs in the fetus, with a lethal outcome.

The natural history of congenital dyserythropoiesis is determined by the severity of the defect and the rate at which iron accumulates and hepatic dysfunction evolves. Most typically, the disease is relentlessly progressive, with only a small percentage of the patients reaching adulthood.

Hematologically, the red cells are macrocytic, although this may change after splenectomy, when normoblasts appear in the circulation and, unusually, stomatocytosis may be present, reflecting abnormalities in the membrane proteins. The bone marrow shows the most striking changes, with erythroblast multinucularity, which is often evident in the spleen.

Ultrastructurally, there is profound distortion of normal cytopathy. These findings are not pathognomonic and, furthermore, the anticipation that they would contribute to understanding the pathophysiology of this disorder has not been fulfilled, although modifications using immunogold electron microscopy may be of more diagnostic value.

Ferrokinetic studies confirm that hematopoiesis is ineffective. When ferrokinetic studies are combined with organ imaging, blood formation is shown to be widely distributed throughout the skeleton.

Use of this combined technique can sometimes identify blood formation in extramedullary hematopoiesis.

Immunologically, information about congenital dyserythropoiesis is largely limited to serologic changes in the acidified serum and sucrose lysis tests, although the pattern varies somewhat with disease subtype. The lesion appears to be due to abnormal complement regulation, at least in type II, and affects constituents other than decay-accelerating factor, known as CD55, or membrane inhibitor of reactive lysis, or CD59. The glycosylation defect that characterizes these cells involves glycoprotein A, which is the major erythrocyte sialoglycoprotein, a known complement regulator. Cold antibody hemoly-
sis may increase, resulting in variable expression of anti-I agglutinins, depending on the subtype being reported.

Biochemically, there is precipitation of beta-globin chains, supporting the concept that an unbalanced assembly of polypeptides makes up the hemoglobin molecule, although the effect of this lesion on the pathogenesis of the disorder is not established. A number of enzymes have been examined, but their diagnostic value has not been confirmed. In contrast, the fatty acid composition of erythrocyte glycosphingolipids is disturbed, particularly in type II patients. Consistent with this finding is an abnormality of both membrane and cytosolic proteins, with the primary defect being failure in glycosylation of substrate by a defective transferase that brings about the membrane lesion.

Clonogenic assays have suggested that erythropoiesis is normal, although ultrastructural studies of the erythroid BFUs showed changes consistent with a defect at the stem cell level. Furthermore, the distribution of erythroblasts within the cell cycle was disrupted, with reduced numbers undergoing DNA synthesis and corresponding increases in the G2 and G1 phases.

Pathogenesis is that of a genetically transmitted defect at the level of the hematopoietic stem cell that results in the biochemical lesions coupled with expression of membrane antigens, one of which has now been designated HEMAP.

Diagnosis is based on morphologic, ultrastructural, and serologic criteria in the peripheral blood and the marrow.

Complications arise from ineffective hematopoiesis, with red cell transfusions accelerating iron overload. In the presence of the erythroid hyperplasia, there is an increased risk of parvovirus infections and, unusually, osteomyelitis may develop.

Management centers on folate supplementation and giving the patient as few red cell transfusions as possible, keeping in mind that the objective of therapy is to keep the patient symptom free. This should be combined with iron chelation to diminish the risks of siderosis. Occasionally, splenectomy has been used, although the results
have been questionable. In rare instances, allogeneic bone marrow transplantation may be considered because it has curative potential, whereas the most realistic future option is replacement of the defective clone by gene transfer.

**WHITE CELLS**

Severe chronic neutropenia encompasses a heterogeneous group of disorders in which there is a selective decrease in circulating cells to levels often associated with recurrent fever, chronic oropharyngeal inflammation, and severe infections. The three major subtypes of severe chronic neutropenia are idiopathic, cyclic, and congenital. Although, traditionally, reduction in numbers has been the criterion for defining this form of bone marrow failure, it may be too narrow a view because qualitative lesions can be lethal, as in severe chemotaxis-induced dysfunction, in which granulocytopenia in the marrow appears normal.

**Chronic Idiopathic Neutropenia**

These disorders are poorly understood, but the obvious defect is chronic hypoplasia in the myeloid lineage that does not affect erythropoiesis or platelet production. No consistent genetic pattern has been reported, but autosomal dominant inheritance has led to familial occurrence among Yemenite Jews.

Historically, information is scanty and mostly anecdotal, with little added after the disorder was first described in 1931.\(^{75}\)

Clinically, the patient is first seen with infection, which is roughly in proportion to the degree of neutropenia, although there are a few serious sequelae.

The natural history is one of low counts, but spontaneous remissions can occur up to the age of 4 years, occurring less frequently thereafter.

Hematologically, the circulating neutrophils are not dysplastic, and the bone marrow shows arrest at the promyelocyte stage, with more mature forms of bone marrow being strikingly absent.

The pathogenesis of chronic idiopathic neutropenia remains unknown, and the defective maturation has not been explained either by demonstrating biochemical abnormalities or through the use of clonogenic assays. Evidence to support ineffective myelopoiesis is scanty.

Diagnosis is made on the basis of persistently very low neutrophil counts that seldom give rise to infections. Except in these circumstances, intervention with corticosteroids, androgens, splenectomy, and cytotoxic drugs would appear to be inappropriate.\(^{74}\) When infection does arise, the use of recombinant human G-CSF is rational.

**Cyclic Neutropenia**

This entity is better described to encompass other lineages because the periodic oscillations, usually occurring every 21 days, also affect erythropoiesis, megakaryopoiesis, and other white cells.\(^{26}\)

Historically, the recognition centered on a report by Leale in 1910 of an infant with recurrent furunculosis. Subsequently, the variants have been classified as being of childhood onset, with or without a family history, and emphasis has been given to distinguishing these from an apparently similar but acquired lesion that has an immunologic basis associated with large granular lymphocytosis.

Clinically, the entity is rather benign, although when counts are low, aphthous ulcers, stomatitis, fever, malaise, and cutaneous or subcutaneous infections may develop.\(^{76}\)

The natural history of the disorder was elucidated after the analysis of hundreds of reports and led to the recognition of three phases.\(^{77}\) The first phase usually lasts 1 to 3 days, with listlessness and irritability occurring. During a second equivalent period, aphthous ulcers appear on the oropharyngeal mucosa, with tender cervical lymphadenopathy. The third phase is a short recovery time.\(^{76}\) Infrequently, there may be associated abdominal pain and diarrhea.

Hematologically, neutropenia fluctuates from normal to very low levels, whereas other cell counts are unaffected or may be slightly raised. Episodes of thrombocytopenia have been reported.\(^{76,77}\) The bone marrow is mildly hypercellular, depending on the stage at which it is examined. When this phenomenon is characterized kineretically, the percentage of cells in the S phase corresponds roughly with circulating numbers.\(^{76}\) Such cyclic variations are also seen in red cell and platelet precursors as well as among lymphoid cells.\(^{77}\)

Clonogenic assays have shown fewer colonies at the time when absolute values are present, although it is not clear how this finding should be interpreted.

Pathogenesis has failed to incriminate an immunologic mechanism. Rather, when human and canine data are combined, a primary defect is seen to lie in early pluripotential cells, thus explaining changes that extend beyond the involvement of the granulocyte series, but the question of genetically determined periodicity remains unanswered. In addition, it is not known whether abnormalities in purine and pyrimidine metabolism documented in the grey collie dog or its clinical counterpart represent an epiphenomenon or may, in some way, point to the basic lesion.\(^{77}\)

Diagnosis of cyclic neutropenia depends on the distinctive sequence of events in hematopoiesis. The first of these is a typical superficial infection that occurs during a neutropenic episode. When this is in the skin, staphylococci and streptococci are responsible, whereas in the mouth, unusual aerobic and anaerobic organisms can
be isolated. There is little evidence that these individuals are particularly prone to viral, fungal, or parasitic infections. Management requires that the patient fully understand the nature of the illness. Although there has been some enthusiasm for the use of recombinant growth factors, these probably have limited value in treatment.

Kostmann's Syndrome

Also known as dysgranulopoietic neutropenia, the syndrome features recurrent infection, absence of neutrophils in the peripheral blood, and bone marrow that shows maturation arrest at the promyelocyte–myelocyte level. Inheritance may be as an autosomal recessive, dominant, or X-linked entity.

Historically, this lethal disease was described in 1956 as agranulocytosis infantalis hereditaria and encompassed multiple abscesses of the skin, as well as otitis, mastoiditis, and high fever resulting from agranulocytosis in a child who lived for 4 months without demonstrable neutrophils. The familial transmission was evident, with half of the eight siblings having died as infants. A similar syndrome had been reported on the father's side, in which all the children except the father died. Once the genetic basis had been recognized, a systematic survey was undertaken in the county of Norrbotten, Sweden, and particularly in the parish of Överkalix, leading to the seminal description of the disease in 1958. Interestingly, the unaffected individuals were normal, and most were typically Nordic, with little evidence to account for Finnish or Lappish ancestry.

Clinically, multiple abscesses develop in spite of the absence of granulocytes, and the distinctive feature is the family distribution; most patients die within 2 months of the onset of their disease, with few surviving beyond half a year.

Hematologically, neutrophils are absent, with the other two lines not directly involved. However, mild degrees of anemia may reflect associated hemolysis and blunted response to erythropoietin, as found in the presence of chronic infection. Reactive thrombocytosis has been recorded. The bone marrow shows striking arrest of maturation at the promyelocyte stage. Ultrastructural and cytochemical studies of neutrophils are consistent with defective formation or degeneration of primary granules, whereas their secondary counterparts are either markedly decreased or cannot be demonstrated at all; autophagia may be striking.

Clonogenic assays, in spite of the presence of neutrophils, show the anticipated numbers of colony-forming cells in both blood and marrow, with an intact maturation sequence.

Pathogenesis of this syndrome, with its dysmorphic neutrophilic granulocytes, ineffective granulopoiesis, negative serology, and absence of antineutrophil antibodies, is in many ways reminiscent of the findings in congenital dyserythropoietic anemia. GM-CSF was unaffected, whereas G-CSF could not be detected, suggesting a defect in the production of the latter stimulatory peptide. This contrasts with reports of supernormal levels, which may be evidence of a blunted response to endogenous levels of the latter ligand. One explanation is a mutation in the gene for the receptor, although this seems to be a relatively uncommon occurrence. Alternatively, because the number of binding sites and affinity are normal, the defect may lie more distally in the signal-transduction pathway, where there is interference with the mobilization of cytosolic free calcium, affecting chemotaxis, superoxide anion generation, and F-actin content.

In individuals with sepsis who have absent or poor wound healing, the diagnosis rests on the presence of severe neutropenia and a family history.

Management consists of local measures and the use of recombinant human G-CSF to restore neutrophil counts, at which point the lesions rapidly reverse. Escalating doses have been advocated, but caution is necessary because empirical use of neutrophils by megakaryocytes, has been observed in some patients, overlapping with the development of thrombocytopenia. Innovative treatment options include combinations with IL-3 or stem cell factor.

Myelokathexis

The association of moderate neutropenia with bizarre morphologic disturbances of granulocyte nuclei, although uncommon, results in intramedullary granulocyte death and can be confused with myelodysplasia. Management centers on the use of growth factors; however, in one case, bone marrow transplantation was successful.

PLATELETS

Thrombocytopenia andAbsent Radii

These patients are seen rarely and have low platelet counts, with bony abnormalities being fairly constant, although cardiac anomalies may occur in one third of these individuals, particularly tetralogy of Fallot and atrial septal defects.

Historically, the syndrome was defined in three families containing nine affected individuals, four unrelated patients, and a survey of 27 previously reported cases. Since then, an increasing number of confirmed reports have appeared.

Clinically, the syndrome occurs typically in children, although it may also develop in later life. Currently, more than 100 examples have been reviewed, resulting in a consolidation of the original features of the disorder and the addition of high-arched palate and abnormalities of the eustachian tract to the list.
The natural history is determined by the severity and duration of the thrombocytopenia, although there is often spontaneous improvement after 1 year of age, and therefore data on patients after puberty are scarce. Hematologically, thrombocytopenia is most severe at diagnosis, and counts fluctuate but tend to rise with age. Bone marrow is hypercellular, with eosinophils prominent and megakaryocytes typically small, immature, and apparently not producing platelets. Iron stores are decreased, reflecting blood loss. Usual associations are abnormalities of chromosome 3 and, possibly, superimposed immune thrombocytopenia. Chromosomal analyses show extreme sensitivity to x-rays, but fragmentation or breaks that typify Fanconi's anemia, which have led to confusion of this diagnosis, are unusual. Clonogenic assays reveal virtual failure of megakaryocytic growth, even in optimally stimulated cultures. Pathogenesis remains uncertain with the defect being absent or with arrested development of committed megakaryocytic progenitor cells in the face of high levels of the appropriate colony-stimulating activity. Diagnosis of the disorder is not difficult in the typical phenotype but may initially be missed when less gross defects are present either in the patient or in family members. Management has been unsatisfactory, with intravenous immunoglobulins recommended and splenectomy sometimes effective. The best results, however, have been obtained with recombinant growth factors or bone marrow transplantation. More recently, the use of IL-3, IL-6, and IL-11 has been advocated to stimulate megakaryopoiesis, but their role is uncertain because benefit is likely to be seen only as long as the stimulator peptide is administered.

CONGENITAL SYNDROMES: EVOLVING TO MARROW APLASIA

INTRODUCTION

Some cytopenias that also have a genetic basis differ from the previously described entities in that initial involvement of the single lineage progresses to global bone marrow failure. These are generally more ominous because of the life-threatening potential; therefore, they pose greater challenges for management.

ERYTHROCYTES

Fanconi's Anemia

Decreasing hemoglobin due to relentless erosion of hematopoietic tissue associated with multiple congenital defects as a typical occurrence in families was recognized more than 50 years ago and was subsequently confirmed. Age, natural history, and skeletal changes vary, and in the Estensen-Dameshek subtype, bone marrow failure may be the sole abnormality. Inheritance is an autosomal recessive with homozygosity producing the typical phenotype, whereas ploidy reduction may be accounted for by interaction with other genes. Contrasting, the heterozygotes have less striking physical and hematologic defects, which is consistent with partial expression of the Fanconi gene. This situation is thought to underlie all cases of acquired aplasia. With clinical expression precipitated by a superimposed environmental insult. This point has been contested, however, in spite of the biochemical lesion remaining obscure, classification is possible by complementation testing, which has identified four separate genes, with the defects localized to at least the long arms of chromosomes 20q and 9q.

Historically, aplasia was described by Erlich in 1888, and in a subsequent review of 64 cases, mental retardation, underdevelopment of the skeleton, and hypogastinalism were added. The syndrome was more precisely defined by Fanconi, after whom it was named. A number of reviews have crystallized the essential features, emphasizing the susceptibility of skin fibroblasts to transformation by oncogenic viruses, thereby raising the specter that chromosomal instability would predispose the patients and their relatives to a variety of malignancies. A consequence of the worldwide interest in this entity is the establishment of a registry at the Rockefeller University, with proposals that it may well be preleukemic and, if so, might provide an experiment of nature for the study of acute myeloblastic leukemia.

Clinically, failure to recognize subtle manifestations leads to underdiagnosis. Low birth weight with growth retardation, morphologic distortion involving mainly the head and face, skin pigmentation, and hypogastinalism, particularly when combined with skeletal abnormalities in the radial ray, should alert pediatricians to this possibility. Further frustration may make diagnosis difficult, and indeed, this entity may be recognized only as a result of hematologic deterioration occurring in pregnancy.

The natural history of Fanconi's anemia is dictated by changes in hematopoiesis, in which irreversible and worsening cytopenia culminates in early death, with survival rarely extending beyond puberty and frequent blood transfusions being complicated by siderosis. A seminal observation was that testosterone and corticosteroids could beneficially influence outcome, and this led to a clinical classification based on disease severity. Of note is the association with acute leukemia and myelodysplasia or solid tumors.

Hematologically, the blood count shows refractory normochromic normocytic anemia and pancytopenia that progresses slowly.
with age. The bone marrow findings differ with the stage of the disease, being normal early on, but gradually, hematopoietic reserve contracts. Erythroagocytosis is not unusual, and transfusional siderosis is striking and may result in secondary hemochromatosis. Radiolabel studies confirm loss of erythropoiesis and, in some cases, superimposed ineffective production. Serologic and immunologic abnormalities are not a feature. Biochemical defects are inconsistent but include the absence of terminal deoxynucleotidyl transferase.

Chromosomal analyses reveal endoreplication and breakage, with the latter more widespread than previously appreciated and associated with defective repair of DNA cross-links. These characteristic lesions can be demonstrated using micronucleus formation techniques, with response enhanced by the addition of mutagenic agents or flow cytometry, which gives information about cell-cycle status. Flow cytometry should be evaluated against the standard approach, in which hypersensitivity of these cells to cytotoxic and clastogenic effects of DNA cross-linking agents is demonstrated using the diethylnlobutane test. It is attractive to suggest that such profound chromosomal aberrations create a milieu that predisposes to rearrangement and subsequent transformation, especially when patients are exposed to low-level environmental mutagens and carcinogens. However, one caveat is that clonal abnormalities need not, in and of themselves, form the basis for high-risk intervention such as bone marrow transplantation, unless further data are provided that clonal abnormalities clearly predict the emergence of leukemia.

Clonogenic assays show reduced growth of erythroid and myeloid progenitors suggesting that there is a defect at the stem cell level. Long-term cultures provide an alternative explanation because hematopoietic progenitors are not only present, but they also have the capacity to proliferate and mature, thereby focusing attention on a potential role for the inductive microenvironment. Thus reduction in early-acting cytokines, specifically stem cell factor, GM-CSF, and IL-6, may have a more influential role than previously appreciated. When stromal cells from patients are examined, output of a variety of growth factors, both constitutively and in response to IL-1, is seen to range from blunting through augmentation in comparison to normal controls, and it is notable that these defects could not be directly attributed to the Fanconi mutations.

Pathogenesis remains controversial. Although not strictly applicable, it is instructive to draw an analogy from two distinct mouse models. Although the two models have a similar phenotype with refractory megaloblastic anemia, absence of mast cells, and changes in their coats, the causative lesion in each is quite different. Mutations in the dominant White Spotting (W) or Steel (S) loci, respectively, prevent expression of a receptor coded by the c-kit oncogene that has homology for a feline transforming retrovirus or its ligand required for clonal growth of early progenitors.

Given this precedent and the lack of a demonstrable biochemical lesion in these patients, it is not unreasonable to remain receptive to the concept that either hematopoietic stem or stromal cells could be primarily responsible for defective synthesis of the protein essential for the recognition, modification, and repair of the characteristic damage to DNA cross-linking. A start has been made in this direction, with complementarity studies that have demonstrated four genes, designated A through D, with one having been cloned and accounting for 15% to 20% of the cases that occur in the United States. However, as in its murine counterpart, it is not clear whether these variants necessarily bring about hypersensitivity to DNA-damaging agents with their predisposition to neoplastic transformation. Furthermore, as an alternative, or perhaps arising concurrently, cellular response to oxidative stress may fail because of a fault in one of the detoxification mechanisms that remove free radicals or their oxygen byproducts. Thus the challenge for experimental hematologists is to determine whether these molecular events are manifestations of a common mutation or whether they differ sufficiently to provide a means for subclassifying affected individuals.

Diagnosis is not difficult when the clinical and hematologic findings are supported by a positive diethylnlobutane test, abnormal micronuclear formation, or a flow cytometric equivalent. Complications are those of the pancytopenia, upon which are superimposed the development of myelodysplasia and acute leukemia.

Management is successful in roughly 50% of the patients given steroids and andabolic androgens as first-line therapy, in the course of which spontaneous remissions may occur. In contrast, experience with the use of lithium or infusion of recombinant human superoxide dismutase is limited. Similarly, administration of erythropoietin or recombinant growth factors has been advocated, but these agents do not have proven value. The preferred approach is allogeneic bone marrow transplantation, using matched siblings, although unrelated donors or umbilical cord vein blood, perhaps with ex vivo expansion, are options. A more definitive treatment awaits cloning of the FancnI gene, at which time direct replacement will have much to recommend it. A fascinating alternative, suggested by the experimental studies, is that intratertine grafting is not beyond our reach.

Dyskeratosis Congenita

This rare form of ectodermal dysplasia, also known as the Zinsser–Engman–Cate or the Cole–Bauschob–Toomey syndrome, is characterized by abnormalities in the skin, with dystrophy of the nails
arising in the first decade and extending to leukoplakia in the second, with age-related progression. The diagnostic triad is reticulated hyperpigmentation of the face, neck, and shoulders; leukoplakia of the mucous membranes; and nail changes. Bone marrow failure is found in half of the patients, usually during the second decade, with cancer developing in the third and fourth decades.

Inheritance is as an X-linked recessive, although other patterns have been reported that are consistent with autosomal transmission or X-inactivation, with this heterogeneity perhaps explicable by genes that are distributed over the X—as well as multiple—autosomes. Lymphocytes in these patients are hypersensitive to bleomycin treatment, which manifests as multiple chromosomal breaks. This phenomenon is a useful test for carrier definition.107

Historically, some 250 patients were originally reported to have poikiloderma atrophicans vasculare, with evidence for a familial occurrence and association with marrow aplasia or Fanconi’s anemia being poorly substantiated. Dyskeratosis congenita is currently considered to be a separate entity, and in the last 25 years, it has been accorded recognition in its own right. Extensive family studies106 have established the sequence in which clinical signs become manifest, beginning with skin pigmentation and nail dystrophy, and culminating in neoplastic changes and opportunistic infection, which lead to early death.

Clinically, the X-linked variant arises in men, although roughly equal gender distribution is found in what appears to be an autosomal-inherited subtype. The median age at presentation is generally 15 to 16 years but ranges from a few weeks to the mid-40s. Skin pigmentation is present in the majority of patients, and oral leukoplakia is found in two thirds. A number of these features simulate chronic GVHD, which has led to the suggestion that both have a similar pathogenesis. The mesodermal damage is genetically determined in dyskeratosis congenita, whereas with the acquired variant, morbidity and mortality typically occur after allogeneic transplantation.104 Other defects include epiphora, cataracts, dysphagia, nasopharyngeal atresia, early loss of teeth, and osteoporosis associated with fractures or aseptic necrosis characterizing the skeletal abnormalities, which are worse in patients being treated with prednisone.

The natural history shows that 10% to 20% of those affected will eventually develop cancer. The lingual hyperkeratosis has a potential for malignant change, which can be demonstrated immunocytochemically. Altered expression of the p53 suppressor gene appears to be a reliable marker for predicting neoplastic transformation in the lesions. The bone marrow fails in between half and three fourths of the cases, although this is generally less frequent in the autosomal variant. Confusion arises with Fanconi’s anemia, but these patients seldom have nail dystrophy, leukoplakia, hyperhidrosis, or hair loss, whereas those with dyskeratosis rarely have chromosomal breaks and appear to develop leukemia less frequently.

Hematologically, the blood count begins as normal, but thrombocytopenia may be the presenting event, and typically there is progressive pancytopenia. The bone marrow is usually hypocellular with radionuclide studies showing a variable distribution of hematopoiesis throughout the axial skeleton.

Immunologically, abnormalities exist in T-cell function, with striking lymphopenia and atrophy of lymphoid parenchyma, consistent with a defect in the cell-mediated immune system. These findings correlate with thymic aplasia and atrophy of the spleen and lymph nodes.108

Chromosomal studies, using appropriate restriction enzymes and probes, map the gene for dyskeratosis congenita to Xq28. Although this disorder is in the general category of chromosome-breakage syndromes, the data are conflicting. Some investigators have reported that findings in lymphocytes and fibroblasts occur spontaneously, with an increased level of sister chromatid exchanges. Other researchers have demonstrated normal values. Such variations can be reconciled if different complementation groups exist to this end. X-irradiation appears capable of revealing fragile sites that may be located for cellular oncogenes and therefore may have diagnostic value in both patients and possible heterozygotes. Skin fibroblasts are ideal for study in culture and have been used to show the marked increase in breaks, hypoploidy, and premature centromere disjunction. Furthermore, banding studies revealed nonrandom lesions that correspond to known breakpoints for cancer-specific rearrangements. Recalling high rearrangement rates occur; particularly in the elderly, an explanation is suggested for the dyskeratosis congenita defect predisposing to these chromosomal changes.

Clonogenic assays show abnormal growth patterns that may be immunologically mediated. However, this mechanism does not exclude the possibility that the stem cell is intrinsically defective. This is supported by data from long-term marrow-culture studies that reveal intact stromal function analogous to the situation in the W/WV mouse and focuses attention on the receptor encoded by the c-kit oncogene as a candidate for the lesion in these patients.110

Pathogenesis centers on the gene for dyskeratosis congenita having been mapped to the X-chromosome, but it is uncertain, from complementation studies, whether other defects may also exist within the genome. Expression results in the characteristic variety of ectodermal lesions. With regard to hematopoesis, the evolution of pancytopenia, based on clonogenic and long-term bone marrow culture assays, is most consistent with a normal microinductive envi-
enronment and a defective hematopoietic stem or progenitor cell. That the lesion is immunologically mediated remains, for the moment, anecdotal.

Diagnosis presents little difficulty when all the physical signs are present. However, this possibility is not always considered when patients are first seen with pancytopenia due to bone marrow failure, malignant change, lingual hyperkeratosis, other malignancies, or the complications arising from their immunocompromised status.

Management remains unsatisfactory, although treatment with recombinant human growth factors has proven beneficial in some instances and less so in others. Currently, bone marrow transplantation offers the best chance for cure. Here a word of caution is appropriate because there are similarities between these individuals and those with Fanconi's anemia, in which chromosome fragility, which is widely distributed throughout all the systems of the body, may be followed by an unusually high incidence of secondary malignancies. Although few innovative options are on the horizon, it is likely that once the genes have been more reliably identified and isolated, the way will be open for their specific replacement.

**WHITE CELLS**

**Shwachman's Syndrome**

Shwachman's syndrome is defined as the familial occurrence of bone marrow dysfunction and pancreatic insufficiency in childhood, with the latter not being due to cystic fibrosis. There are associated abnormalities at the metaphases of long bones, ichthyotic skin rash, hepatosplenomegaly, cardiomyopathy, frequent infections, and developmental retardation. Genetically, studies are relatively limited and show no consistent abnormality.

Historically, six children were described. The children had diarrhea, weight loss, and low levels of trypsin, lipase, and amylase. The distinctive feature, however, was normal electrolyte levels in the sweat. Four of the children had unexplained iron-resistant anemia, and all had intermittent or persistent neutropenia and thrombocytopenia. Another similarity was copper deficiency, and indeed, it was suggested that this was a contributing factor when associated with pregnancy. Over the years, very little new information has been added other than neurologic assessment, which has shown a reduced intelligence quotient, hypotonia, deafness, and retinitis pigmentosa, with sibship segregation ratios supporting an autosomal recessive mode of inheritance.

Clinical presentation is that of children who, in the first year of life, fail to thrive due to diarrhea, steatorrhea, weight loss, and eczema, with a differential diagnosis of cystic fibrosis. Aggregation among family members is evident soon after the index case is identified. Of note is just how many tissues and organs are involved, ranging from dental abnormalities, renal dysfunction, and endocrine disturbances, such as a delay in puberty or diabetes mellitus, to a variety of dysmorphic features. Unusually, there may arise central pontine myelinolysis, pancreatic lipomatosis, and respiratory dysfunction. Skeletal abnormalities are osteoporosis, in which copper deficiency could be incriminated. The outcome is typically determined by the severity of the marrow failure.

Hematologically, varying degrees of pancytopenia are present, with low neutrophil count generally detected in the first 5 years of life. Raised hemoglobin F levels may precede bone marrow function, and hypocellularity is usually found.

Ultrastructurally, cells reveal the Pelger–Huet anomaly, with dilated endoplasmic reticulum containing granules that look like intracellular inclusions. These could represent failure to secrete a normal product or may possibly reflect synthesis of an abnormal protein.

Immunologically, impaired function has been linked to aberrant phagocytosis and defective chemotaxis. Superimposed on this, however, is the suboptimal activity of natural killer cells, which may contribute to the recurrent infections. The latter report is somewhat at odds with previous descriptions, in which humoral and cell-mediated mechanisms were found to be intact.

Chromosomal analyses show increased spontaneous breakage that broadly links these children with those having Fanconi's anemia and dyskeratosis congenita.

Clonogenic assays have been carried out infrequently, but results are usually in the normal range.

Pathogenesis is uncertain but is consistent with a transient, albeit severe, copper deficiency in early childhood. Although multiple organs are involved, giving rise to protean manifestations, some insight into the causation derives from studies on isolated chondrocytes, in which large glycogen and small lipid deposits suggest that the basic defect resides in the function of microfilamentous elements. These findings become more prominent when the cultures are exposed to colchicine, which favors an intrinsic inability to repair the microtubules; this would be particularly damaging in active tissues such as the marrow.

Diagnosis is not difficult when a malnourished child is seen with hypoplastic pancytopenia and is found to have metaphyseal chondroplasia and a history of recurrent infection; confirmation is derived from a defect in neutrophil locomotion and natural killer cell activity.

Complications are numerous and involve the respiratory, gastrointestinal, hepatic, renal, and neurologic systems. A word of caution is
appropriate because many of these young children have features reminiscent of cystic fibrosis, although this possibility can generally be excluded.

Management is largely symptomatic, with the infections responding promptly to antibiotics. The morphologic abnormalities in the gastrointestinal tract are reversible with simple enteral nutrition. Lithium has been reported to benefit neutrophil function and does this without side effects, perhaps by improving the cytoskeletal defects. However, the current vogue favors the use of cyclosporin A. Probably the most efficient way to deal with the infection problem is by parenteral administration of recombinant human G-CSF. Bone marrow transplantation is clearly effective, although the data are largely anecdotal.

Reticular Dyogenesis

The coexistence of congenital agranulocytosis, lymphopenia, and the absence of cellular or humoral immunity is also known as thymic lymphopenia or aleukocytosis. No genetic defects have been demonstrated and, in spite of an excess number of males affected, this is not accepted as an X-linked disorder.

Historically, the first cases were reported in 1959, but few, typically aggregated in families, appear to have been described thereafter.

Clinically, infection dominates early infancy and the striking feature is failure of lymphoid tissue to develop in nodes or tonsils, with the thymus not demonstrable on chest radiograph or computed tomography (CT) scan. The severe granulocytopenia combines with immunologic paralysis to render the outlook extremely grave.

Hematologically, the counts are low and granulocytes are absent, resulting in a relative lymphocytosis; anemia is frequent, but thrombocytopenia is unusual. The marrow is hypocellular, with myeloid and lymphoid precursors being scanty. Normoblasts, although reduced, may be dysplastic.

Immunologically, there is an absence of T and B lymphocytes associated with a low immunoglobulin level and profoundly suppressed response to mitogens; biochemistry is normal.

Clonogenic assays have not been helpful, with colonies and clusters being reduced in size and number when compared to controls.

The pathogenesis is unknown and currently is speculated to reflect a defect in stem cells that are incapable of undergoing lympho-myeloid differentiation.

Diagnosis, if this syndrome is considered, is confirmed without difficulty, but the rapid progression of the disease leads to early death from overwhelming infection.

Management depends on early recognition of this possibility, and a successful outcome may follow allogeneic bone marrow transplantation.

PLATELETS

A megakaryocytic thrombocytopenia

A low platelet count in the infant, extending to the other two lineages, is an infrequent but well-recognized entity, although fewer than 50 cases appear to have been reported. Conceivably, many synonyms have diluted the distribution of reports in the literature, including congenital essential thrombocytopenia, familial thrombocytopenic purpura, isolated megakaryocytopenia, congenital hypoplastic thrombocytopenia with malformations, type III constitutional aplastic anemia, and megakaryocytic thrombocytopenia. A genetic basis has not been established, but at least some cases appear to be X-linked, and others have autosomal recessive transmission.

Historically, the infrequency with which cases have been described over many years may explain the lack of uniform diagnostic criteria. However, this does appear to run in families, with reports from Australia, suggesting the existence of male and female subgroups, designated type III and type IV. Such gender distribution is questionable, however. A preferable approach is to emphasize the presence or absence of physical anomalies.

Clinically, the dominant feature is bleeding into the skin, mucous membranes, or gut, with the majority of individuals progressing to marrow aplasia. Thereafter, children fall into two broad categories, with the common finding being bone marrow megakaryocytes that are either reduced or cannot be demonstrated at all. Specifically, all patients have a normal radial ray. Separation is based on those who have no associated organ abnormalities, in contrast to the remainder, who do have associated organ abnormalities. When these abnormalities are present, they are not characteristic of any other recognizable syndrome. Examples include defects of the central nervous system, the presence of cardiac disease, distorted facies, and retarded development.

The natural history is a median survival of 6 years in patients with no associated organ abnormalities, but only 2 years in those with associated abnormalities. Mean ages at death from thrombocytopenia were 1.9 and 0.5 years, respectively, and from aplasia, 8 and 2.7 years, respectively. In spite of the poor outlook, an extensive review of all reported cases has shown that a few patients may survive beyond childhood.

Hematologically, thrombocytopenia with macrocytosis is initially present, whereas bone marrow cellularity is unaffected except for a decrease in or absence of megakaryocytes, which, nevertheless, appear morphologically normal. Interestingly, there are increased levels of hemoglobin F and expression of the i antigen, which are two indices of significant bone marrow damage. Radionuclide investigations show unimpaired survival of homologous platelets, so that the low
counts are due to underproduction, which is consistent with loss of the corresponding marrow precursors. Accelerated peripheral sequestration is not usually found, although there is anecdotal reference to the presence of immune complexes in the serum, hinting at an underlying immunologic abnormality.

Chromosomal analyses demonstrate increased numbers of breaks and gaps, but contrary findings have also been reported. Clonogenic assays have not shown a decrease in megakaryocyte progenitors, but few cultures appear to have been carried out during the thrombocytopenic stage. Once aplasia has developed, overall hematopoiesis is profoundly reduced.

Pathogenesis remains unknown, but because congenital aplasia may first involve this lineage, these patients could conceivably reflect the initial events in the latter syndrome, which then extends to the other two lines. The diagnosis is suggested by thrombocytopenic bleeding at an early age, in the absence of bone marrow megakaryocytes.

Management requires the control of hemorrhage and infection, which remain the most common causes of death. Steroids are effective in about one third of patients and, when combined with androgens, can lead to partial response, whereas splenectomy is without value, and there are no long-term survivors. Given this poor outlook, attempts to base therapy with stimulatory peptides on the in vitro responses that occur with IL-3 and GM-CSF were reasonable, but the assays turned out not to have any predictive value. More recently, stem cell factor has become available, but inadequate data are available to comment on its usefulness in these rare individuals. Perhaps the most reasonable option, where indicated, is still autologous.

FAMILIAL MARROW DYSFUNCTION

A surprisingly large number of patients, having the common finding of aplasia that apparently occurs in families, do not fit comfortably into any of the recognized syndromes. An interesting approach is to group these patients on the basis of transmission, with the caveat that substantial overlap occurs with spontaneous mutations.

Autosomal Dominant

Examples of autosomal dominant familial marrow dysfunction include the oculo-oto-radial syndrome, with mild thrombocytopenia, leukocytosis, radial hypoplasia, and deafness. Closely allied is the entity described as WT that occurred in two families, who had abnormalities of the hand and subsequently developed leukemia. In others, there has been coexistent pigmentation, immunodeficiency, and vascular disease in the presence of neurologic dysfunction, culminating in ataxia. All of these have superficial similarity to the aplasia described by Fanconi but fail to meet the minimum diagnostic criteria of chromosomal breaks and gaps. Modern techniques should, however, make it easier to identify and classify correctly individuals with chromosomal gaps because peripheral blood and lymphocyte responses to diphenylbutane stress testing is now a standard procedure.

Autosomal Recessive

In a number of these patients, immunodeficiency occurs together with hematopoietic failure. A relatively wide range of possibilities has been described, and at least some have distinctive chromosomal abnormalities, including monosomy 7.

X-linked Recessive

Families have been identified in whom infectious mononucleosis or another viral related hemophagocytic syndrome gives rise to distinctive morphologic findings in the marrow. In one example, males over a number of generations apparently developed acute myeloblastic leukemia and light chain disease. As with any large pediatric experience, disturbed hematopoiesis occurring without stigmata in any other organ system, particularly when this is aggregated in families (even though it is sometimes limited to a single generation), raises a question about exposure to a common environmental mutagen.

Miscellaneous, with Associated Skeletal Abnormalities

There are odd examples of aplasia developing in children with physical defects that are not acceptable as variants of the syndrome described by Fanconi. These include Friedrich's ataxia, in which patients have short stature and hypogonadism and children who have webbed neck and abnormalities of the thumbs, but even these appear to vary widely within the individual family.

RARE HEMATOLOGIC SYNDROMES

Differing degrees of bone marrow failure have been associated with trisomy 21, or Down syndrome, in which there is transient neonatal myeloproliferation and a risk of leukemia in later life, although only a few of these individuals ever develop aplasia. Similar eponymous descriptions are those of Dubowitz and Seckel, with their individual distinctive growth defects, hematopoietic disorders, and malignancy.
ACQUIRED DEFECTS: PERSISTENT SINGLE LINEAGE

INTRODUCTION

Isolated anemia, leukopenia, or thrombocytopenia, which, by definition, are variants of bone marrow failure and therefore justify brief comment, may be late presentations of congenital syndromes, which in adults are more often immunologically mediated. The marrow is hypercellular, although there may be selective loss of one or another lineage, and typically there is left-shift in the marrow precursor pool, with residual progenitor growth in appropriate clonogenic assays.

ERYTHROCYTES

Pure Red Cell Aplasia (PRCA)

Anemia with absent reticulocytes and loss of the normoblast population has a number of causes, most of which have an immune basis and occur without abnormalities in the granulopoiesis or thrombocytosis; familial occurrence excludes this diagnosis. Historically, description of PRCA in 1922 by Kazzol was initially followed by confusion with the Josephs-Diamond-Blackfan syndrome, and a number of other terms emerged, including erythropoiesis, progressive postinfectious erythroblastosis, isolated aplastic anemia, anerythropoietic anemia, chronic erythropoietic hyperplasia, and primary pure red cell aplasia, but much of this experience appears to be anecdotal. Clinically, presentation is with anemia; symptoms and signs are determined by individual compensatory mechanisms.

The natural history identifies a small category of unknown origin and pathogenesis. In a further idiopathic group, pathologic immune globulins are found; these are directed either against normoblasts or, alternatively, against erythropoietin. The remainder of cases are secondary to associated disease (Table 4). Hematologically, there is normochromic normocytic anemia, which may occasionally be macrocytic and severe. Bone marrow typically has increased cellularity due to the persistence of granulocytes and megakaryocytes, but the loss of nucleated red cells is striking, and this arrest may occur at a particular stage. For example, if the target is intermediate normoblast, then the earlier basophilic forms remain abundantly present. The Coombs' test is negative, but during the hemolytic phase, lactate dehydrogenase and bilirubin may be transiently elevated.

Immunologically, a wide range of associations has been reported. In the primary group, an offending antibody can often be isolated. Others have the lesion attributable to dysfunctional lymphocytes, which may have a T-gamma phenotype and exert profound immunosuppressive effects, or, alternatively, may be a prominent, large, granular population with natural killer cell activity. Occasionally, such cytotoxicity appears to reside in less clearly defined mononuclear fractions.

Clonogenic assays demonstrate T-cell-mediated damage to erythroid precursors, with other cell lines in the GEMM-CFU assay being normal. Pathogenesis appears to be primarily through clonal expansion of T lymphocytes. Precedent from animal studies suggests that viral infections can generate a transmembrane glycoprotein capable of impeding erythropoiesis by interference with interaction between ligand and receptor or subsequent signal-transduction pathways. Diagnosis rests on the findings of distinctive peripheral blood and marrow morphology.

Management has seen erythropoietin largely replace red cell transfusions to control symptomatic anemia. The underlying immune lesion generally responds to prednisone, which is often given in escalated doses or combined with cyclosporin; other options are conventional cytotoxic drugs, antilymphocyte globulin and high-dose gamma globulin.

Transient Erythroblastopenia of Childhood

The distinctive feature is temporary reticulocytopenia and varying degrees of anemia, with absence of erythroblasts in the marrow, whereas white cell precursors and megakaryocytes are preserved.
Clinically, a previously healthy child, typically in the third year of life, is seen with profound anemia after a viral infection of the upper respiratory or gastrointestinal tract, often associated with seasonal clustering.

Hematologically, the red cell count is reduced and reticulocytes cannot be demonstrated, but the remainder of the count is normal, although thrombocytopenia and neutropenia are sometimes seen. Erythroblasts are absent from the marrow. During the recovery phase, children manifest stress erythropoiesis, releasing a cohort of fetal-like red cells because hemoglobin F levels are increased and expressing a antigen, but this is a transient phenomenon.

Pathogenesis is sometimes unknown, but in the majority of these children, evidence of parvovirus infection can be demonstrated. Management is conservative, with red cell transfusions occasionally needed, whereas intervention, as with prednisone, may generally be avoided.

**WHITE CELLS**

**Agranulocytosis**

Chronic idiopathic immunoneutropenia has parallels with lesions that may arise in red cells and platelets on an immunologic basis. The synonyms over the years have included congenital or genetic agranulocytosis and hypoplastic neutropenia.

The first description by Moeschlin goes back some 40 years, although the immune nature and the benign course have been recognized more recently. Clinically, although this disorder may occur in children, the average presenting age is 30 years, with a 3 to 1 female preponderance. No family history has been demonstrated. More than half the affected individuals had been exposed to agents known to cause neutropenia, but their withdrawal seldom altered the neutrophil count. Nevertheless, it is important to be sure that there has been no contact with cytotoxic or antithyroid drugs and the antplatelet agent, ticlopidine, which may cause damage in sensitized individuals. Of interest in this regard is the retention of toxic metabolites such as arene oxides, which can normally be broken down but accumulate in predisposed hosts that have microsomal abnormalities and suppress the marrow. Similarly, it is important to identify and exclude from consideration in this category individuals in whom an immune mechanism is secondary to Sjögren's or Felty's syndrome in the course of rheumatoid arthritis. Although infection occurred in 27% of the patients, typically when absolute counts were less than 0.5 × 10^9/L, there was a surprisingly low incidence of serious morbidity, attributed to compensation from humoral immunity involving lymphocytes, monocytes, and the recruitment of marginated neutrophils. In this context, there has been one case of enhanced serum bactericidal activity, but follow-up studies are lacking.

Hematologically, there are varying degrees of neutropenia, whereas the marrow has reduced myeloid to erythroid ratios, with the reserves of more mature neutrophils being decreased, as judged by lack of response to endotoxin, etiocholanolone, cortisone, and epinephrine. These static observations are substantiated by radionuclide kinetics, showing that free and margined cells, as well as turnover rates, were decreased, but interestingly, the half-life in the peripheral circulation was approximately normal. The way in which such changes arise remains controversial, with some evidence that production is reduced and marrow compensatory efforts are insufficient to correct the neutropenia. Other information supports adequate neutrophil release under conditions of stress but subnormal delivery in the basal state.

Immunologically, the neutropenia is primarily mediated by autoreactive or alloreactive neutrophil-specific antibodies of unknown origin. Although some studies have failed to demonstrate these, oligoclonal immunoglobulins can be incriminated, both quantitatively and functionally. Interestingly, there is light chain restriction of these granulocyte-binding proteins that probably does not reflect malignancy but suggests a somatic mutation. In a subset of patients, antibody titers are higher and combinations of IgG and IgM occur, with some of the latter fixing complement. This gives rise to additional cytopenias, associated splenomegaly, and more infections. Less clear-cut are individuals in whom inhibitory immunoglobulins are directed against precursor cells, whereas immune complex formation may provide yet another mechanism in certain individuals. Furthermore, expansion of a CD8⁺ lymphocyte subpopulation has been reported in a few of these patients, suggesting that widely differing etiologic mechanisms may result in the chronic immune neutropenias.

Clonogenic assays are generally normal, although in a single case, autologous plasma inhibited growth of both the patient's mononuclear cells and a heterologous target population. This finding is reminiscent of red cell aplasia, with an antibody having its major effect against committed progenitors.

Pathogenesis is probably variable, with the idiopathic neutropenia being due to an inhibitory autologous immunoglobulin in at least some cases. In others, an expanded CD8⁺ population is consistent with cell-mediated direct cytotoxicity that may be associated with prominent large granular lymphocytes. These can, on occasion, be leukemic, although the molecular defect may be via the elaboration of inhibitory cytokines.

Diagnosis is that of otherwise unexplained neutropenia, usually
with few complications, implying that significant compensatory mechanisms are effective.

Management requires the exclusion of nonbenign variants, and thereafter the patient needs to understand the nature of his or her disease, with the safest course being mastery inactivity. If infectious episodes occur and do not respond promptly to antibiotics, recombinant human growth factors are of clear benefit.

**PLATELETS**

Amegakaryocytic Thrombocytopenia

In most adults, unexplained and often profound reduction in platelet count occurs on an immune basis, with abundant megakaryocytes demonstrable, although there is unequivocal evidence that in some of these individuals, the marrow precursors may disappear; this is more common than was generally appreciated. A similar finding has been described with the ingestion of drugs and alcohol.

Historically, in patients with an isolated loss of megakaryocytes, the diagnosis has been confused with this hematologic abnormality, which occurs as a congenital or constitutional variant, and sometimes has overlapping associations such as immunoglobulin deficiency or the Wiskott–Aldrich syndrome. Currently, this notation should be reserved for individuals with an intrinsic defect at the level of the megakaryocytic progenitor or a circulating cytotoxic autoantibody directed against the corresponding CFU.

Clinically, patients with isolated thrombocytopenia, even with purpura, may be seen with anemia due to gastrointestinal tract blood loss. In these patients, the differential diagnosis includes immune mechanisms, with both hereditary or acquired marrow aplasia requiring exclusion, as do myelodysplasia and systemic lupus erythematosus. It should be recalled that a number of these cases may progress to global marrow failure, representing forms of bone marrow lesions typically associated with absent radii or reticular dysgenesis. The cyclic variant occasionally causes confusion, but the oscillating nature of the platelet count should resolve the dilemma. It appears that antibodies may selectively block the action of growth factors whose receptors are found on megakaryocytic progenitor, and peripheral blood mononuclear cells may periodically suppress megakaryocytic precursors. Rare correlations are with vitamin B₁₂ deficiency and certain anemias.

Hematologically, the blood count is normal apart from thrombocytopenia, whereas the marrow shows complete or partial absence of the large precursors.

Immunologically, antiplatelet antibodies may be directed against common antigens on the megakaryocyte membrane or, infre-

to their loss from the marrow. Occasionally an immunoglobulin may be generated that selectively blocks the action of GM-CSF, which, apart from its action on granulocytes and macrophages, affects these progenitors. Interestingly, determination of plasma glycocalicin may help with classification. Thus its significant reduction correlates with an absence of megakaryocytes, but platelet life span is unaffected. In contrast, normal levels of this fragment of glycoprotein Ib are found where thrombocytopenia results from immune-mediated peripheral sequestration; there is no overlap of values between these two groups.

Chromosomal analyses have shown an association with duplication of the long arm of chromosome 3, but no acquired clonal defects appear to have been reported.

Pathogenesis remains uncertain; this is probably a syndrome with diverse etiologies. In some, there is an intrinsic defect at the level of the progenitor, and in others, a comparable result is brought about by circulating cytotoxic antibodies or cell-mediated suppression of megakaryocytopenia.

Diagnosis is contingent on excluding all of the diseases that may lead to loss of precursors, most often through immunologic mechanisms. Of these, the lymphoproliferative disorders are sometimes overlooked, and it is worth stressing the correlation with systemic lupus erythematosus, particularly in population groups in which this disorder is frequently encountered.

Management centers on reversing the underlying mechanism, and remissions have been obtained with antithymocyte globulin. However, if thrombocytopenia is sufficient to cause bleeding, allogeneic platelets are of value in the short term, although their repeated infusion may result in immunization. Although tranexamic acid is reported to be of little benefit, our experience is that it may be helpful, particularly as a means of reducing the amount of replacement therapy needed. As a general rule, cytokines such as IL-3, -6, and -11 are of little benefit, and bone marrow transplantation, given the currently recognized causative mechanisms, is not a viable option.

**ACQUIRED DEFECTS: EVOLVING TO MARROW APLASIA**

**INTRODUCTION**

In previously normal individuals, the sudden and irreversible loss of hematopoietic cells is a surprisingly common and potentially lethal event. The tradition of referring to this as aplastic anemia has little to recommend it because, rather than selecting erythropoiesis, all cell lines are affected. It is even less logical to define a subcategory as being very severe on the basis of granulocyte counts; the more accurate and therefore preferable term is marrow anaplasia. An
additional confounding practice is the inclusion of varying levels of pancytopenia, reflecting hypoplasia outside the diagnostic criteria, in discussions of this entity. As a result, many reports attribute inflated response rates to a particular type of intervention. In contrast, it seems more scientifically sensible to ensure that only those patients meeting the internationally agreed definition form the basis for any study, although lesser degrees of damage can be accommodated in trials by proper stratification. In the vast majority of cases and in spite of a diligent search for causation, no exposure to recognized agents such as viral infection, which are increasingly important in acquired immunodeficiency disease, irradiation, or drugs can be uncovered. Such acquired idiopathic destruction of the blood-forming organ may, nevertheless, reflect the subtle impact of a critically damaging environmental insult on a genetically predisposed marrow. Clinical and experimental evidence supports the viewpoint that the final common pathway involves immunologic mechanisms, placing the majority of these patients in the broad category of autoimmune disease. However, the possibility of microenvironmental dysfunction should not be overlooked because, although not strictly applicable, precedent from anemic mouse strains has established that the identical phenotype may arise from mutations affecting distinct loci, which produce molecular lesions in either stem cell or stroma. It is also intellectually stimulating to speculate with the postulate that morphologic dysplasia and clonal hematopoiesis in the patient with aplastic anemia may be part of a spectrum that includes a hypoplastic variant of the classic myelodysplastic syndromes.

IDIOPATHIC MARROW APLASIA

Severe pancytopenia, defined as granulocytes and platelets less than 0.5 x 10^9/L and 20 x 10^9/L, with less than 1% reticulocytes when singly corrected for hemocrit, coupled with a bone marrow either less than 25% of normal cellularity or up to 50% of normal, provided more than 70% of the remaining cells are nonhematopoietic, remain the internationally accepted criteria for diagnosis. Recommendations to modify these criteria on the basis of different reticulocyte counts have had little support. Similarly, by definition, aplasia cannot have grades of severity. This uncritical terminology has crept in to explain varying outcomes associated with the use of antilymphocyte globulin (ALG) and should preferably be discarded or, at best, restricted to use in this specific context. It has no relevance in patients eligible for bone marrow transplantation. Additional prerequisites are the absence of any predisposing factor and exclusion of a congenital variant, although genetic heterozygosity predisposing to marrow failure may be difficult or impossible to demonstrate.

Equally subtle is the potential influence of certain class II histocompatibility antigens that identify a fragile marrow that is exquisitely sensitive to chloramphenicol and, by analogy, other less clearly defined environmental insults. These postulates are supported by the marked increase in RNA transcripts for the stem cell inhibitor gene in some of these patients, in whom relatively high expression in unstimulated lymphocytes suggests a mechanism for cytotoxic suppression through the arrest of progenitor cell cycling. Thus, although most cases remain idiopathic, the use of molecular techniques is steadily forging new links between seed and soil.

Historically, the association of fatty atrophy of the marrow with cytopenia should be attributed to Erdich, but the term aplastic anemia seems to have been first used by Chauffard in 1904. As the congenital syndromes were identified, it became possible to exclude them from this entity, although it was not until cellularity could be examined during life that the relationship between the peripheral blood and this compartment was clarified. A further advance was the shift away from pure morphology to a more dynamic approach that introduced new terms in the form of stem cells, hematopoietic micro-environment, and regulatory mechanisms. Almost immediately, it was appreciated that aplasia was the final common pathway for a number of insults, some perhaps on the basis of genetic susceptibility and others associated with immunologic disturbances. Such seminal observations underlie progress made in the last 20 years and have drawn support from refinement in animal experiments. These have been strengthened by the introduction of new technologies, prominent among which are culture systems. Last, and of particular importance, have been the results of immunologic manipulation and, indeed, bone marrow transplantation in patient management.

Clinically, global bone marrow failure is reflected in three major symptoms, of which anemia is usually prominent. Additionally, there is easy bruising or purpura, and neutropenia gives rise to sinopulmonary and urinary tract infections. Findings are otherwise unremarkable and, importantly, skeletal or vascular malformations that characterize the constitutional counterparts of this disease are absent. In recent years, this pattern has been modified by coexistent infection with the human immunodeficiency virus, so that hairy leukoplakia, oropharyngeal candidiasis, and even Pneumocystis carinii pneumonia may be early symptoms.

The natural history of idiopathic marrow aplasia in the untreated patient is one of rapid progression, with median survival in the range of 4 months, and only approximately 10% alive at 1 year, and even then the survival curve has not reached a plateau. Two additional facets are notable. First, epidemiology varies widely among countries because statistics are difficult to compile as a result of both poor data recording and a paucity of information but on death certificates. Even
using the international classification for diseases is not infallible because those individuals with the genetic variant are misdiagnosed and included in this category. Nevertheless, the incidence appears to be low in France whereas estimates for the United States are between two and five cases per million annually, with the result that these individuals will be encountered by most practicing physicians during their careers, often in the setting of prior drug therapy. Second, many features are shared between idiopathic aplasia and the myelodysplastic syndrome (MDS) or other clonal disorders of hematopoiesis, including paroxysmal nocturnal hemoglobinuria (PNH). This overlap assumes special relevance in patients receiving biologic immune response modulation with ALG, which is often given in combination with other agents, such as corticosteroids, in whom the longer survival is associated with the increased occurrence of MDS, PNH, and acute leukemia. 

Hematologically, variably variable pancytopenia is associated with a surprising degree of dyserythropoiesis in the form of macrocytosis, megaloblastic maturation, hypogranular neutrophils with Pelger-Huet anomaly of the nucleus, and platelets that may be large and stain abnormally. This morphology is reminiscent of classic myelodysplasia. The marrow findings differ with the stage of the disease. Typically, there is little recognizable hematopoietic tissue and prominence of adipocytes, with a scattering or lymphocytes and plasma cells. Paradoxically, there may be areas of intense blood formation, known as "hot spots." An additional feature is severe atrophy, which may be associated with acute inflammatory changes or myelitis, and is most evident during the early stages of the disease. Radiouclide kinetics are important to quantify residual hematopoiesis. Characteristically, clearance of the labeled iron is markedly prolonged, and effective red cell use is virtually absent. Such erythrocytes are as formed have a short survival, and external scanning shows virtual absence of isotope uptake in areas normally associated with blood formation; instead, accumulation is in the liver, spleen, and other reticuloendothelial tissues. Serologic studies are of little value other than in characterizing patients who have PNH. Positive acidified Ham's acidified serum lysine and sucrose tests can be used for this purpose. In this context, decay-accelerating factor and CD59 deficient populations, typically limited to the red cells, may first appear in granulocytes and monocytes.

Immunologically, the idiopathic variant is unremarkable apart from mild reduction in lymphocytes and monocytes or modest depression of plasma immunoglobulin levels, although no consistent differences from normal have been reported. The significance of circulating immune complexes remains controversial. Conversely, although not related to pathogenesis, profound changes arise in the patient with aplastic anemia when it is secondary to systemic lupus erythematosus or acquired immunodeficiency disease.

Biochemically, there is an increase in hemoglobin F, reflecting reversion to a fetal type of hemoglobin. A rare family defect in cellular folate uptake has been said to cause severe aplasia that reversed on treatment with folic acid. Chromosomal analysis is often apparently normal, but abnormalities may be uncovered in those individuals with clonal hematopoiesis. In children, this has been demonstrated by the patterns of X-linked genes. Furthermore, lymphocytes have increased strand breaks in DNA supporting the contention that some individuals may have either forms frustes or heterozygous variants of Fanconi's anemia. Conceptually, this situation can be regarded as functional hypoplasia, in which residual stem cells have a reduced capacity for self-renewal that fails when they are exposed to some otherwise trivial insult.

Clonogenic assays have shown a deficiency of early stem cells, whereas suppression in growth is mediated by T cells or immunoglobulins. Most of the evidence, including cross-culture experiments from my laboratory, is consistent with these patients having a normal hematopoietic microenvironment. In contrast is a report that, at least in some Chinese patients who have received prior chloramphenicol, there is also demonstrable stromal damage. A defect at this level could adversely affect production of IL-1, with a consequential decrease in release of CSFs from fibroblasts and endothelial so that the hematopoietic progenitors fail to grow. Similarly, IL-3 generation by mononuclear cells is significantly lower in these patients, correlates with disease severity, and has been postulated to be a predictor of outcome. A comparable situation has been reported with the ligand for the c-kit receptor. Attempts to dissect pathogenesis further using stromal cell lines have, to date, been of only limited value. Nevertheless, there is precedent derived from murine models in which a common phenotype, albeit one that is limited to anemia rather than to marrow aplasia, can result from a defect in the stem cell or the stroma. It follows that because an immune-mediated lesion may operate at either level, it would be prudent to keep an open mind to the possibility that, at least in some cases, the defect may lie primarily in the hematopoietic-inductive microenvironment. In support of this theory, we have recently shown that patients who have morphologically normal peripheral blood and bone marrow and are undergoing transplantation nevertheless still have a profound reduction in the clonogenic capacity of their CD34 progenitors; importantly and in parallel, the stroma becomes more slowly confluent whereas, in cross-culture experiments, it is functionally subnormal (Novitzky N, Jacobs P, unpublished).
Pathogenesis in its simplest clinical context represents pancytopenia with chronic fatty atrophy of the red marrow. Experimental hematologists favor a more dynamic stimulation, in which output from this tissue is inadequate to meet even basal trilineage requirements. From either standpoint, aplasia is seen as the common expression for a variety of lesions that may arise at any point in the complex pathway needed to produce mature cells. Understanding this is helped by recalling the progenitor cell model (Fig. 1).

Conceptually, stem cells may be constitutionally predisposed or primed for bone marrow failure by the complement of genes they carry. Inheritance could, therefore, create a fragile population that need progress no further but that is susceptible to environmental insults that may be subtle and unrecognized, so that self-replication fails and hematopoiesis is lost.

Even if one accepts such an intrinsic defect of the hematopoietic tissue, the mechanism leading to overt damage remains speculative, but accumulating clinical and experimental evidence favors a role for the immune system. Support is found in the occurrence of hypogammaglobulinemia, reports of antibody-mediated aplasia, and a range of cytotoxic phenomena residing in lymphocytes or in the monocytes and macrophages. Such immunologic mechanisms have been demonstrated in a wealth of data from culture studies.

A further attraction of this postulated autoimmunity is that it refocuses attention on the inseparable interdependence of stem cells and the microenvironment, from which there is reduced growth factor production or local generation of negative regulators such as gamma interferon. It also gives credence to the suggestion that the many defects in stromal function, although not necessarily immediately causative, play a significant and often aggravating role in the pathogenesis of aplasia. Undoubtedly, however, we have not heard the last word on this topic.

Diagnosis presents little difficulty, and here it need only be emphasized that adherence to internationally accepted criteria is mandatory if proper classification is to be the basis for optimum treatment and stratification to trials.

Management is dictated by a high index of clinical suspicion, followed immediately by confirmatory marrow examination and avoidance, at all costs, of well-intentioned delays in anticipation that spontaneous recovery will occur. Specifically, multiple red cell and platelet transfusions are to be discouraged because immunization adversely affects the outcome after transplantation. Similarly, protracted and inappropriate administration of erythropoietin or recombinant growth factors is not rational, because, in controlled trials, they do no more than transiently raise counts without altering the natural history of the disease with contrasting experience being anecdotal.

Yet another important caveat is that hypoplasia, best quantitated by combining trephine biopsy with ferrokinetic studies, is excluded from this discussion because such patients will generally respond to androgenic and steroids. In the individual in whom aplasia has been correctly defined, matched sibling allogeneic bone marrow transplantation is the treatment of choice, particularly in children younger than 5 years. In these patients, immunosuppressive therapy produces very poor results. Outcome after grafting has been improved by the use of recombinant human growth factors, which have relatively few side effects and in controlled clinical trials have reduced morbidity and cost. Although platelet recovery with G-CSF and GM-CSF has been slow, this is likely to be reversed by the use of IL-3, -6, and -11, perhaps in conjunction with early-acting cytokines such as the stem cell factor. Since the first description, the principles of bone marrow transplantation have been largely standardized. Infusion of fractionated marrow is preceded by conditioning with cyclophosphamide as a single agent and is followed by graft rejection in up to 50% of patients, with little benefit derived from further conditioning with total nodal irradiation. It is noteworthy that in some of these individuals, autologous recovery may nevertheless occur, as reflected in their becoming chimeras.

The technique used for aspiration is important, and the rate of engraftment is best when the largest number of clonogenic precursors are obtained. After infusion, clonogenic precursors home to marrow niches or sometimes to extramedullary sites, as determined by recognition systems with galactosyl and mannose specificities. Approximately 14 days later, discrete clusters of hematopoietic tissues are evident. In most cases, these are polyclonal, but occasionally monoclonality exists, thereby proving that, under normal circumstances, single stem cells are able to repopulate both lymphoid and myeloid compartments. Of interest is the continuing debate about the origin of stromal cells, including the endothelium. Similarly, the role of adhesion molecules, and particularly, the influence of heparan sulfate in the matrix as determinants for engraftment are important areas in which further information is needed.

Morphologic reconstitution is generally easy to recognize, although early on it may also be present in the spleen, suggesting that substantial injury to the marrow has taken place during conditioning. This is consistent with the abnormal production of cytokines during this period. Furthermore, neutrophils may be dysfunctional and thus contribute to infection during the recovery phase. To some extent, these deficiencies can be overcome by quantitatively optimizing the graft and attempting to avoid exposure to agents that, applicable to autologous but not allografting, may damage the precursors.

Turning attention now to immunologic recovery, there are three
major issues. The first is that of graft failure or subsequent rejection, and here pancytopenias are found, especially when there is a mismatch between donor and recipient in ABO or Rh systems. This may be T-cell mediated and, in an HLA-DR restricted way, a specific subset that is CD3+, HNK1+, CD8+, and DR+, appears to be either in- 
hibited or responsible. Of relevance is that this particular phenotype is present at extremely low concentrations, but even so, has the requisite inhibitory capacity, which is perhaps related to the exquis- 
ite sensitivity of these lymphocytes being able to recognize host differ-
ences as small as a single amino acid. Second, recovery of immu-
nity is variable and requires vigilance to treat aggressively infections when they arise and, in the longer term, to consider suitable vacci-
nation programs. Third is the unique phenomenon of acute or 
chronic GvHD, in which incoming lymphocytes destroy skin kerati-
nocytes, biliary endothelium, and enterocytes, predominantly in the 
small bowel. The morbidity and mortality of the more severe 
grades, designated II through IV, are prohibitive and span a variety 
of immunosuppressive regimens. Initially this was based on a canine 
model, documenting benefit from methotrexate, although more re-
cent additions have included combinations with cyclosporin and 
and/or prednisone. In spite of a reduction in the incidence and severity of 
both acute and chronic GvHD, these remain major complications and 
and have led to the exploration of alternative approaches, primarily in the 
form of T-cell depletion. In most cases, monoclonal antibodies were 
administered to patients singly or as cocktails, with uneven results.

Although morbidity from GvHD diminished, this was offset by an 
increased incidence of graft loss. When these recipients also received 
Campath 1G intravenously both before and after the procedure, graft 
loss appears to be prevented. In our patients with aplastic anemia, 
graft rejection was initially encountered when first transplanted and a number of individuals required one or more retransplants. Our 
numbers are small; however, our outcome differs significantly from 
that of a much larger experience reported by the Aplastic Anemia Working Party. The addition of the same monoclonal antibody, 
given to the recipient around the time of the procedure, has over-
come this limitation. It is noteworthy that in this context, no other 
immunosuppressive agents are used. The discrepancy between some 
of the published results and our own may relate to our inclusion of 
total irradiation as a routine procedure. Although concerns have been 
raised about the use of this modality in patients who do not have 
malignant disease, they do not appear, for the moment, to be suffi-
cient to discontinue the practice.

When there is no suitable sibling to donate bone marrow, alternatives include the use of marrow from partially matched donors or 
from a matched, unrelated source. These approaches, however, carry a much higher incidence of rejection and GVHD. Nevertheless,
The use of ALG has been advocated as first-line therapy, but there are a number of caveats. Its outcome is less satisfactory when given to young girls. Furthermore, there is an association with the later development of clonal or malignant disease, including myelodysplasia, although the abnormal clone may disappear. Indeed, the same hematologic lesions may be present in patients who never received this form of treatment. Further complication is the occurrence of PNH, in which a complement-regulating glycoprotein renders erythrocytes exquisitely sensitive to lysis. Molecular techniques have established that these patients develop a glycosylphosphatidylinositol-anchoring defect, and if PNH were to be defined by this criterion, its incidence would probably be much higher. Furthermore, those with this molecular defect are likely to have a less good outcome when given immunosuppressive therapy. The link between PNH and myelodysplasia is controversial also because the preleukemia is thought by some to have arisen in the PNH clone and by others to derive separately from the injured marrow. Of note is the demonstration that a concurrent deficiency exists for the urokinase-type plasminogen activator receptor, which may account for the high incidence of venous thrombosis in these patients.

A further point is the relatively low response rate to ALG when it is used alone, and it should not be forgotten that this product leads to the development of serum sickness, in which severe arthralgia may be present. In an attempt to modulate these symptoms and to obtain an additional immunosuppressive effect, ALG was given with high-dose methylprednisolone. Given together, these drugs produce responses exceeding 70%, but the dose of steroid may well not be critical. A combination of ALG and anabolic androgens is effective beyond 4 months, particularly in female patients with low neutrophil counts. Unfortunately, this treatment has little benefit on short-term survival. The role of testosterone is additionally helpful in that it may reduce the amount of complement sensitivity and so perhaps influence outcome in those destined to develop PNH. Because cyclosporin is approximately equivalent to ALG with steroids, these three agents were used in a single regimen, with apparent benefit. However, the low response rates for their control group, for example, when compared to the Cape Town data, raise a question about the adequacy of the reference arm in the study by Frickhofen et al. When extended to a multicenter trial, it was shown that cyclosporin alone was approximately equivalent to ALG and steroids.

The critical study remains to be done. This would use ALG of proven efficacy with either standard or high-dose methylprednisolone, and patients would be randomized to receive an addition of cyclosporin or anabolic androgens. Until these data become available, the relative effect of the latter agents remains an open issue. For centers that have small patient numbers and therefore are unable to embark on randomized trials, it would be reasonable to join a multicenter study or to use the four drugs together, as is our current practice.

No discussion on the contemporary management of bone marrow failure would be complete without reference to the role of interleukins and stimulatory peptides. Anecdotal suggestions of bilineage response rests on long-term administration of the recombinant granulocyte growth-facilitating molecule to adults and children, although much larger than conventional doses may be needed. Additionally, the administration of stem cell factor provides results that, at least in vitro, are consistent with the defects residing at stem cell level. This contrasts with the failure to obtain benefit in the constitutional variants, such as that described by Fanconi, in which there is an intrinsic lesion of progenitor cells. In view of its early action in the differentiation pathway, this particular molecule, if it has any effect at all, is anticipated to exert this synergistically when given in a cocktail.

SECONDARY MARROW APLASIA

Systemic Lupus Erythematous

The classic syndrome is idiopathic and is considered to be autoimmune, in spite of hints at a viral etiology. A closely allied entity occurs with the administration of procainamide and prazotol, with other agents sometimes implicated. For the most part, hematologic defects are limited to peripheral blood and take the form of cytopenias. Only very rarely has aplasia or amegakaryocytic thrombocytopenia been reported.

Viral Infections

Hepatitis A typically causes mild leukopenia and thrombocytopenia, but aplasia has been recognized for some 40 years and, based on syngeneic allograft data, an autoimmune reaction is postulated. Hepatitis B was first implicated in 1978, with in vitro studies demonstrating infection of human bone marrow cells, although a causative mechanism remained obscure. The same syndrome has been documented in the non-A, non-B infection and may be transient or irreversible; aplasia has been reported after orthotopic liver transplantation for this particular viral disease. Currently, involvement by the type C virus is increasingly diagnosed. Hepatitis C is responsible in at least some cases and at present is probably underdiagnosed. In all of these individuals, the final common pathway is conjectural, but immunologic defects are thought to play a significant role, with opportunities to study pathogenesis in those areas such as Taiwan, where frequencies are high. Epstein-Barr virus has been reported to destroy hematopoiesis, as
has rubella, although much more rarely. Parvovirus typically produces a transient aplastic crisis, which may, on occasion, be persistent and irreversible.

The human immunodeficiency virus gives rise to red cell aplasia, but more ominously, this may extend to involve the whole marrow. The worldwide impact of this epidemic has led to extensive studies on hematopoiesis, with cytopenias being most common and attributable to abnormalities in humoral and cell-mediated pathways, in which the myelosuppressive effects of current treatment regimens may well aggravate the symptoms. Frustratingly, attempts to improve outcome with erythropoietin or a variety of growth factors have been, at best, transient.

Human herpesvirus 6 can cause chronic bone marrow suppression, but more often than not, chronic bone marrow suppression occurs concurrently in patients with acquired immunodeficiency disease.

**DRUGS**

Blood dyscrasias can be ascribed to many different and commonly used agents, although, not infrequently, their administration is unrecognized. In a number of cases, these cause aplasia but, fortunately, death seems to be relatively rare or underreported.

Chloramphenicol consistently damages the marrow if dosage is excessive, and there is little reason for this to occur. In contrast, a rare idiosyncratic variant in some individuals causes irreversible hematopoietic destruction and has a high mortality rate, with such genetically predisposed individuals difficult to anticipate, and even eye drops containing a small amount of chloramphenicol are sufficient to cause this lesion. The erythrocytic changes are well recognized and correlate with the rate at which a test dose of the antibiotic is cleared from the plasma. The first abnormalities are ultrastructurally demonstrated vacuoles in the pronormoblasts and immature myeloid cells, but these soon become evident on light microscopy. It is interesting that the clinical syndrome frequently has a period of jaundice that antedates the hematologic abnormalities and may lead to the mistaken diagnosis of viral hepatitis. The intraerythrocytic metabolic pathways are affected not only by the parent substance, chloramphenicol, but also by its derivatives, including thiaphenicol. These lesions have been demonstrated using marrow-culture studies or by examining the aminolevulinic acid synthetase pathway in rabbit reticulocytes. Chloramphenicol remains an excellent drug; but it is important to appreciate the risks, which can be significantly reduced by keeping within the prescribed total dose and concurrently monitoring patients with serum iron and percentage saturation of transferrin.

The H₂ receptor blockers, first in the form of metiamide, produced **TABLE 5. Miscellaneous aplasia-producing agents**

<table>
<thead>
<tr>
<th>Nonsteroidal antiinflammatory drugs</th>
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<tbody>
<tr>
<td>Sulindac</td>
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<tr>
<td>Indomethacin</td>
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<tr>
<td>Naproxen</td>
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<tr>
<td>Fenoprofen</td>
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<tr>
<td>Piroxicam</td>
</tr>
<tr>
<td>Indoprofen</td>
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<tr>
<td>Alleprinil</td>
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<tr>
<td>Antiarrhythmic drugs</td>
</tr>
<tr>
<td>Tocainide</td>
</tr>
<tr>
<td>Captopril</td>
</tr>
<tr>
<td>Enalapril</td>
</tr>
<tr>
<td>Antiplatelet drugs</td>
</tr>
<tr>
<td>Thiolipidive</td>
</tr>
<tr>
<td>Analgesics</td>
</tr>
<tr>
<td>Corticosteroids</td>
</tr>
<tr>
<td>Distalgesic</td>
</tr>
<tr>
<td>Anticonvulsant drugs</td>
</tr>
<tr>
<td>Moxsudin</td>
</tr>
<tr>
<td>Other drugs</td>
</tr>
<tr>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Dapsone</td>
</tr>
<tr>
<td>Mesalazine</td>
</tr>
<tr>
<td>Chlorpheniramine</td>
</tr>
<tr>
<td>Rubber cement</td>
</tr>
<tr>
<td>Methyleneketone exposure</td>
</tr>
<tr>
<td>Antituberculosis therapy</td>
</tr>
<tr>
<td>Radiophosphorus overdose</td>
</tr>
<tr>
<td>Azathioprine</td>
</tr>
<tr>
<td>High-dose chemotherapy</td>
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<td>Gammagide</td>
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<tr>
<td>Mefloquine</td>
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<tr>
<td>Methyprylon</td>
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<tr>
<td>Aminoglutethimide</td>
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<tr>
<td>Ethofoximide</td>
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</table>

*In many cases, the evidence is poorly documented and anecdotal. Nevertheless, when prescribing any form of medication, the clinician should be aware of dose-related constraints, even genetically determined idiosyncratic constraints are impossible to anticipate.*

granulocytopenia and led to withdrawal of the agent. However, cimetidine and ranitidine are also capable of suppressing the marrow and may lead to agranulocytosis. It appears that these drugs act directly on the hematopoietic precursors, although irreversible marrow failure seems to be uncommon.

The antithymic drugs exemplified by azathioprine...
thimazol, cause aplasia. Such marrow damage should not arise with proper dosage and, because the damage is immunologically mediated, it is usually reversible.

Many other medications and an assortment of environmental insults have also been incriminated in the production of bone marrow failure (Table 5).

SUMMARY AND CONCLUSIONS

Any imbalance between requirements for mature blood cells and their supply, whether at the basal level or after stimulation by stress, is defined as bone marrow failure. Bone marrow failure can be thought of as a spectrum ranging from subtle alterations within a single lineage to gross and life-threatening pancytopenia due to aplasia. When all of the variants are considered, this entity occurs rather frequently. It follows that all practitioners need to remain vigilant to such a possibility in their patients and, once it is considered, can confirm the diagnosis by the use of relatively simple investigations. Thereafter, it is important to initiate appropriate supportive care and, should spontaneous resolution not take place, to plan for allogeneic bone marrow transplantation.

Because manifestations are protean, the different syndromes often appear at first sight to be unrelated. Each syndrome represents breakdown at a particular point on a common hematopoietic pathway that can usefully be described as the progenitor cell model.

Such a pathophysiologic approach is helpful because it begins by focusing on the peripheral blood and the marrow examination—two sets of morphologic criteria with which most physicians are familiar. From this vantage point, clonogenic assays and long-term culture techniques have revealed the influence of stimulatory peptides and their counterbalancing negative regulators. Both sets of molecules are generated by the stroma or the inductive microenvironment and are presented to their cognate receptors, which are expressed on the surface of hematopoietic progenitors. These membrane glycoproteins then transduce the signal through a series of cytoplasmic substrates to the nucleus. Some of these steps are, for the moment, obscure, but they lead to synthesis of a transcription factor that has a particular conformation or motif that binds to DNA. In this way, genes are activated, and their products direct further cellular function. Options range from entering the cycle to proliferate and mature, diversion to G₀, or programmed cell death. These fundamentals can be used to understand and classify clinical problems.

The first major subdivision is between those having constitutional or acquired defects. Of those with constitutional defects, some will manifest no further than to involve a single lineage in these patients, it is a matter of experience and fine judgment as to what intervention, if any, is appropriate. In others, there may be relentless progression to aplasia so that replacement of the defective organ is unavoidable.

In contrast, otherwise healthy individuals may be seen with anemia, leukopenia, or thrombocytopenia and a relatively normal marrow. In many instances, an immunologic mechanism is operative, exemplified by hemolytic anemia, drug-mediated agranulocytosis, or HIV-associated thrombocytopenia. This category is included in our broad definition for the sake of completeness.

Another group of patients considered to have idiopathic disease are those in whom global marrow function may be profoundly impaired, as with cytotoxic drug therapy, but in whom recovery is the rule. Untoward or unanticipated prolongation in the pancytopenia may occasionally be found, but care in a reverse-isolation unit coupled with judicious antibiotic administration is all that is required. It should be emphasized that only rarely will it be necessary to resort to the use of recombinant human growth factors or interleukins. It is mandatory that these valuable pharmacologic agents not be abused, as this is cost-ineffective and tarnishes their deservedly good reputation.

Penultimately, aplasia may arise suddenly and inexplicably. Once it is clear that spontaneous remission will not occur, allogeneic bone marrow transplantation is the treatment of choice. In some circumstances, this will not be possible, and although response can be obtained with antilymphocyte globulin in combination with immunosuppressive drugs and anabolic androgens, this is not always complete and may be complicated by the emergence of clonal hematopoiesis, including leukemia.

Two concluding comments are appropriate. First, when all these variants are considered, it is surprising how little attention this important topic has attracted. Nevertheless, order and a sensible classification can be imposed on the many eponymously characterized entities, based simply on defining the molecular level at which hematopoietic regulation is disrupted. This information allows a well-reasoned and cost-effective algorithm to be developed for management. Second, it is fitting that very different experimental approaches, carried out over decades, should start fitting together like the parts of a jigsaw puzzle to provide a solid foundation for clinical practice, with the beneficiary of these collaborative efforts being our patients.

ACKNOWLEDGMENTS

I thank Christine Dilling for her very great assistance in compiling the bibliography, Wayne Jacobs for the art work, and Jackie Davies for her dedication and professional help in the preparation and typing of this manuscript.
DEDICATION

Dedicated to Dr. E. Donnall Thomas, Nobel Laureate, for his sustained leadership in this field, and to the late Dr. Mortimer M. Bortin for his vision in establishing the International Bone Marrow Transplant Registry.

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Severe Aplastic Anemia: A Prospective Study of the Effect of Early Marrow Transplantation on Acute Mortality

By Bruce M. Camitta, E. Donall Thomas, David G. Nether, George Santos, E. C. Gordon-Smith, Robert P. Gale, Joel M. Rappeport, and Rainer Starb

A prospective randomized trial of therapy for severe aplastic anemia was designed to compare early bone marrow transplantation with conventional treatments. All patients with a sibling matched at the major histocompatibility region were transplanted. Transplantation was performed within 17–100 (median 33) days of original diagnosis. Conventional treatments included transfusion support with or without androgens. Twenty-four of 36 patients entered on the transplant arm are alive after 4–20 (median 9) mo with full marrow reconstitution. Only two are limited by chronic graft-versus-host disease. In contrast only 12 of 31 conventionally treated patients are alive. Six of these survivors have improved, five incompletely. The 19 nontransplant deaths have occurred within 1–11 (median 3) mo of diagnosis. Compared to nontransplant regimens, early transplantation more effectively restores normal marrow function and decreases the acute mortality of severe marrow aplasia (p = 0.006). Pending longer follow-up, early marrow transplantation appears to be the most effective available treatment for severe aplastic anemia.

The appropriate treatment for aplastic anemia remains controversial. An effect of androgens in marrow aplasia was reported in 1961. However, despite several confirmatory studies, other investigators have been unable to demonstrate the efficacy of anabolic agents. These conflicting results were probably due to the small size of some series and to the use of historical rather than concurrent controls. In addition, patients were not uniformly stratified according to the severity of their presenting disease. When such stratification was performed, it was found that patients with severe disease usually had a short lethal course regardless of treatment. In contrast, patients with milder aplasia often had prolonged courses with more frequent responses to androgens.

Recent reports indicate that bone marrow transplantation from a histocompatible donor is effective therapy for severe aplastic anemia. Most patients have been transplanted after apparent failure of androgens and transfusion support. Delay of transplantation predisposes to morbidity from infection and hemorrhage and increases the chances of patient sensitization to blood products or to donor transplantation antigens. Thus, transplantation earlier in the course of severe aplasia, before failure of conventional treatments, might improve the chances of success.

This paper was prepared for the International Aplastic Anemia Study Group. See the Appendix for participating centers and grant support.

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Blood, Vol. 48, No. 1 (July), 1976
In order to determine the relative merits of different therapies for severe aplastic anemia, a cooperative prospective randomized trial was designed. The objectives were (1) to compare the efficacy of early bone marrow transplantation with more conventional treatment, and (2) to evaluate (in patients given transfusion support) the role of androgens in treatment of severe marrow aplasia. This communication reports results relevant to the first question.

MATERIALS AND METHODS

Definition

To qualify as severely aplastic, patients had to have at least two of the following three peripheral blood values: (1) granulocytes < 500/cu mm (2) platelets < 20,000/cu mm and (3) reticulocytes < 1% (corrected for hematocrit). In addition the marrow had to be either markedly hypoplastic (< 25% of normal cellularity) or moderately hypoplastic (25%-30% of normal cellularity with < 30% of remaining cells being hematopoietic) as estimated from biopsies.

Histocompatibility Typing

As soon as possible after diagnosis the patient and all members of his/her family were typed for the serologically detected HLA-A and HLA-B antigens. Cells from HLA-A/HLA-B matched siblings were then studied with patient cells in mixed leukocyte culture (MLC). Non-reactivity confirmed matching at the HLA-D locus. All donors were compatible with recipients in these tests.

Eligibility and Patient Randomization

Only newly diagnosed cases of severe aplastic anemia were eligible for this study. Patients were observed for 10 days to determine the presence of an underlying illness, to establish baseline blood counts, to detect early spontaneous hematologic improvement, and to complete histocompatibility typing. During this time prednisone (10 mg/sq m/day) was given, in an attempt to detect underlying leukemia.21 Patients with pancytopenia due to nutritional deficiency, malignancy, preleukemia, myelofibrosis, or Fanconi's anemia and patients with other life-threatening diseases were excluded from the study.

The outline of the protocol is given in Fig. 1. All patients with matched siblings were scheduled to receive a marrow transplant. Patients without matched siblings were randomized to one of three arms: (1) no androgen, (2) oral androgen (oxymetholone), or (3) intramuscular androgens (nandrolone decanoate).

![Fig. 1. Randomization: See text for neontransplant rerandomization criteria.](image)
Transplantation Procedures

Protocols for pretransplant immunosuppression are given in Fig. 2. The rationales for and details of doses and schedules used have been previously published. Cyclophosphamide (CY) was used alone for conditioning for engraftment, except in patients thought to be presensitized to their donors or who had rejected a prior marrow graft. Antithymocyte serum could be used in place of antithymocyte globulin (+).

Patient Care

Transplanted and nontransplanted patients were given transfusions of blood components as required. HLA-matched products were utilized for refractory patients (when available). All patients were watched carefully for infection and treated with appropriate antibiotics when infection occurred. Nontransplanted cases were managed as outpatients except for the frequent complications. Prednisone, 10 mg/kg/day, was continued in a few nontransplanted cases at the discretion of the patient’s physician. Isolation procedures for transplanted patients varied from simple reverse precautions to laminar air flow isolation with sterile diet and gut sterilization depending on institutional facilities.

Criteria for Improvement

Complete response was defined as the return of all blood counts to normal values. Partial response meant improvement so that the patient no longer qualified for severe status and no longer required transfusions.

Informed Consent

Informed consent of the patient, the donors, and the involved family members was obtained utilizing procedures and consent forms approved by the ethical review committees of the various institutions involved.

RESULTS

The transplanted and nontransplanted patients were compared for factors felt to have prognostic significance in aplastic anemia (Table 1). The groups were comparable except for four patients over the age of 45 in the nontransplant group.
Table 1. Pretreatment Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Transplanted</th>
<th>Nontransplanted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>16 (1–43)*</td>
<td>13 (0.3–77)</td>
</tr>
<tr>
<td>Male/female</td>
<td>16/17</td>
<td>21/10</td>
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<tr>
<td>Etiology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>26</td>
<td>23</td>
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<tr>
<td>Postinfection</td>
<td>4</td>
<td>4</td>
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<tr>
<td>Drug-induced</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Insecticide</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>Interval of symptoms–diagnosis (wk)</td>
<td>3 (0–17)</td>
<td>3 (1–17)</td>
</tr>
</tbody>
</table>

Initial hematologic values

<table>
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<tr>
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<th>Transplanted</th>
<th>Nontransplanted</th>
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</thead>
<tbody>
<tr>
<td>Platelets/cu mm</td>
<td>6 x 10^2 (1–23)</td>
<td>5 x 10^9 (1–25)</td>
</tr>
<tr>
<td>Reticulocytes (%)†</td>
<td>0.1 (&lt;0.1–1.4)</td>
<td>0.3 (&lt;0.1–1.5)</td>
</tr>
<tr>
<td>Nonmyeloid marrow cells (%)</td>
<td>85 (40–99)</td>
<td>90 (30–99)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses represent the range of values.
†Corrected for hematocrit.

Transplanted Patients

Thirty-six patients entered the transplant arm during the first year of the study (Table 2). Twenty-six received CY alone and ten CY plus ATG and PCB in preparation for engraftment. Although the protocol specified that pretransplant immunosuppression begin by day 21, suppression usually began later (median 33, range 12–97 days). These delays were due to practical problems involved in diagnosis, histocompatibility typing, informed consent, and (not infrequently) waiting for an available bed at a transplantation center.

Two patients died of sepsis during pretransplant immunosuppression. The remaining 34 patients were successfully engrafted. Twenty-four of these 34 (67%) of all patients entered on the transplant arm are now alive with complete marrow restoration after 4–20 (median 9) mo (Table 3). Two long-term survivors are physically limited by severe chronic GVHD. The others returned to regular activities within 3–12 mo after transplantation. In this limited number of patients, there was no correlation of transplant success with patient age or sex, etiology of aplasia, transplant center, or transplantation regimen.

Table 2. Outcome of Transplants

<table>
<thead>
<tr>
<th>Number</th>
<th>Event</th>
</tr>
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<tbody>
<tr>
<td>36</td>
<td>2 Died during immunosuppression</td>
</tr>
<tr>
<td>34</td>
<td>4 Died, infection</td>
</tr>
<tr>
<td>2</td>
<td>Sepsis, GVHD (day 63, 133)</td>
</tr>
<tr>
<td>1</td>
<td>Pneumonia, sepsis (day 89)</td>
</tr>
<tr>
<td>1</td>
<td>Tuberculosis (day 140)</td>
</tr>
<tr>
<td>9</td>
<td>Graft rejection</td>
</tr>
<tr>
<td>1</td>
<td>Died before retransplantation</td>
</tr>
<tr>
<td>1</td>
<td>Spontaneous recovery</td>
</tr>
<tr>
<td>7</td>
<td>Retransplanted</td>
</tr>
<tr>
<td>5</td>
<td>Died, no take</td>
</tr>
<tr>
<td>2</td>
<td>Engrafted</td>
</tr>
<tr>
<td>24</td>
<td>Alive with full marrow recovery</td>
</tr>
</tbody>
</table>

CAMITTA ET AL.
Eight patients rejected their grafts within 4-5 wk after transplantation. Another lost marrow activity during adenine arabinoside therapy begun 23 days post-transplantation for disseminated Herpes zoster. One of these nine died before retransplantation could be attempted. A second patient developed increasing granulocytes during retransplant immunosuppression with ATG and PCB. CY and marrow were not administered. Complete autologous (documented by sex chromosome analysis) marrow recovery ensued. Seven patients who rejected their initial grafts were retransplanted with two successes.

**Nontransplanted Patients**

Thirty-one patients were assigned to nontransplant regimens: 7 NO, 17 PO, and 7 IM androgens. Results to date have been similar in these small groups and they are reported together (Table 3). Seven patients improved, (one complete and six partial), but one partial response was only transient. Responses began from 1 to 3 mo after randomization. Each responder showed improvement in all three cell lines. Compared to transplanted patients, the response rate in nontransplanted patients was significantly decreased (p < 0.001, Wilcoxon).

Nineteen nontransplanted patients died. Time to death was 1.11 (median 3) mo after randomization. The cause of death was hemorrhage in ten, sepsis in seven, and a combination of sepsis plus hemorrhage in two. Figure 3 is a life-table plot of survival of transplanted and nontransplanted patients. Compared with transplanted patients, mortality in nontransplanted patients was significantly increased (p = 0.006, Wilcoxon).

![Fig. 3. Life table plot of the effect of treatment on survival in severe aplastic anemia. Triangles indicate duration of follow-up of current survivors.](image-url)
Nonrandomized Patients

Two patients improved before randomization. One relapsed after 2 mo and died of hemorrhage. The second relapsed after 4 mo and remains aplastic.

DISCUSSION

The present study has confirmed the short lethal course of most patients with severe aplastic anemia when treated with conventional nontransplant regimens. In contrast, mortality in transplanted individuals was significantly decreased. Although we did not evaluate early versus late transplantation, the results reported here were superior to the prior series in which most patients were transplanted after failure of conventional treatment. It would appear that the small chance of early recovery on conventional therapy was probably more than offset by the morbidity, mortality, and sensitization by transfusion that delay must entail.

This study did not evaluate the role of intensive support in the management of severe aplastic anemia. Marrow transplant patients require intensive support during pretransplant immunosuppression and during the 2–3-wk period of total aplasia before the transplanted marrow begins to function. Some of our transplanted cases were managed in protected environments and received oral non-absorbable antibiotics. Such regimens have decreased morbidity in myelosuppressed leukemic patients, but their value in a transplant setting is not yet established. However, since hematologic recovery in severe aplasia is usually slow, if it occurs at all, short periods of isolation would probably have little effect on eventual mortality of nontransplanted patients. The physical, financial, and personnel resources required for trials of long-term intensive environmental support of large numbers of nontransplanted severely aplastic patients are not currently available.

The better outcome of our transplanted patients might also be attributed to superior granulocyte and platelet transfusion support available at transplant centers. However, nontransplanted individuals managed at transplant centers fared no better than similar patients treated elsewhere. However, it is still possible that mortality in nontransplanted severe aplastic anemia could be decreased by wider availability of HLA matched nonfamily blood product support.

The importance of long-term follow-up of surviving patients with aplastic anemia must be emphasized. Patients who recover after conventional treatment may relapse and die months or years later. Similarly, although promising in the short-term management of severe aplasia, transplantation may have adverse long-term sequelae that have not yet been encountered. Nevertheless, in the Seattle series of 23 aplastic anemia patients who survived more than 1 yr after allogeneic marrow transplantation, only one patient has died, one has chronic GVHD, and one has almost completely recovered from chronic GVHD. The other 20 patients are in good health with normal marrow function after 2 to 4 yr follow-up.

Despite the above caveats, this study shows that prompt bone marrow transplantation significantly decreases the early mortality of severe aplastic anemia.
That finding in itself is an important reason for application of this complex treatment in a devastating disease.

REFERENCES

APPENDIX: CONTRIBUTING INSTITUTIONS AND PHYSICIANS

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<td>A. Paciotti</td>
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<td>Yale University, New Haven</td>
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<tr>
<td>West Side V.A. Hospital, Chicago</td>
<td>R. Epstein, W. Fried</td>
<td></td>
</tr>
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</table>

We would also like to thank the following physicians for referring cases to us: C. Abidgaard, A. Alter, R. Berberich, J. Bondi, C. Corley, J. Day, Q. DeMarco, L. Ellis, D. Forque, G. Gilchrist, J. Hales, J. Hersman, H. Messmermore, J. Phillips, J. Reed, Y. Sax, L. Sieger, and L. Zelkowitz.
Effectiveness of Immunosuppressive Therapy in Older Patients with Aplastic Anemia

André Tichelli, MD; Gérard Socié, MD; Michel Henry-Amar, MD; Judith Marsh, MD; Jakob Passweg, MD; Hubert Schrenzmeier, MD; Shaun McCann, MD; Jill Hows, MD; Per Liujman, MD; Pedro Martin, MD; Aruna Raghavachar, MD; Anna Luceaciuili, MD; Alois Gratwohl, MD; and Andrea Bacigalupo, MD, for the European Group for Blood and Marrow Transplantation Severe Aplastic Anaemia Working Party

Background: Immunosuppressive therapy has been used for successful treatment of severe aplastic anemia, but little information is available on outcome in older patients.

Objective: To evaluate outcome in patients older than 50 years of age who received immunosuppressive therapy for aplastic anemia.

Design: Retrospective cohort study.

Setting: 56 centers of the European Group for Blood and Marrow Transplantation (EBMT).

Patients: 810 patients with aplastic anemia reported between 1974 and 1997. Patients were evaluated according to age group: 60 years of age or older (n = 127), 50 to 59 years of age (n = 115), and 20 to 49 years of age (n = 568; reference group).

Intervention: Antilymphocyte globulin, cyclosporine, or both.

Measurements: Survival, cause of death, response to treatment, relapse rate, and risk for late complications were analyzed in all patients and by age group.

Results: The 5-year survival rate was 57% (95% CI, 46% to 66%) in patients 50 to 59 years of age and 50% (CI, 39% to 60%) in patients 60 years of age or older compared with 72% (CI, 58% to 76%) in patients younger than 50 years of age (P < 0.001). Response to therapy, relapse rate, and risk for clonal complications were similar in all three age groups (P > 0.2). Age was significantly associated with an increased risk for death (relative risk compared with patients 20 to 49 years of age, 1.80 [CI, 1.29 to 2.52] for patients 50 to 59 years of age and 2.57 [CI, 1.87 to 3.53] for patients ≥ 60 years of age), mainly because of bleeding or infection (P = 0.02). Response to immunosuppressive therapy in all patients at 12 months was 62% (CI, 58% to 66%); no difference was seen among the age groups in multivariate analysis (P > 0.2). Sixty-six of the 379 responding patients (17%) subsequently had relapse. The risk for clonal disorders at 10 years was 20% (CI, 15% to 27%).

Conclusions: Response to immunosuppression in aplastic anemia is independent of age, but treatment is associated with increased mortality in older patients.

Aplastic anemia is defined as peripheral blood pancytopenia associated with unexplained hypopcellularity of the bone marrow without excess of blast cells. If aplastic anemia goes untreated, patients die of bleeding or infections caused by aplasia. Bone marrow transplantation and immunosuppressive treatment have improved outcome, with remission rates of 60% to 80% (1–4). The decision between immunosuppressive therapy and bone marrow transplantation depends largely on the availability of a bone marrow donor.

In many centers, the upper age limit for allogeneic bone marrow transplantation in patients with aplastic anemia has been set at 40 to 55 years (5–8). This limit is traditionally less stringent for immunosuppressive treatment (2, 9–14). However, data are scarce, and no study has specifically addressed outcomes in older patients with aplastic anemia. After immunosuppressive treatment, hematologic recovery is slow and often incomplete (15), and clonal transformations, such as myelodysplastic syndromes, paroxysmal nocturnal hemoglobinuria, or solid tumor, may occur (16–21). As a consequence, fear of life-threatening complications during prolonged aplasia as well as concerns about increased risk for clonal transformations, particularly in older patients, prevail.

We sought to 1) determine the outcome of patients 50 years of age or older receiving immunosuppressive therapy for aplastic anemia and 2) investigate the response and complication rate among these patients compared with that of younger patients.

Methods

Design

This retrospective cohort study used data from 56 centers reporting to the European Group for Blood and Marrow Transplantation (EBMT) Severe Aplastic Anaemia Working Party between 1974 and 1997. Collected data included demographic information, pretreatment blood values, type of immunosuppressive therapy, date and number of courses of immunosuppressive therapy, response to therapy, date of last known vital status, cause of death, and


For author affiliations and current author addresses, see end of text.

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type and date of late complications for every patient. Follow-up was completed by June 1997.

Patients

We included 810 patients from the EBMT Registry in whom acquired severe aplastic anemia was diagnosed according to current criteria (22), adequate immunosuppressive therapy (antilymphocyte globulin, cyclosporine, or both) was instituted, and bone marrow transplantation was not performed as second-line treatment. For the purpose of this analysis, patients were separated into three age groups: 50 to 59 years of age (n = 115), 60 years of age or older (n = 127), and 20 to 49 years of age (n = 568). The latter patients served as the reference group. The Severe Aplastic Anemia Working Party regularly stipulates the definitions for disease and late complications to its participants. No central slide review was performed.

Outcome Measures

Outcome measures analyzed were overall survival, causes of death, response to immunosuppressive therapy, rate of relapse in responders, and late complications. Late complications were defined as secondary development of a myelodysplastic syndrome, leukemia, paroxysmal nocturnal hemoglobinuria, or solid tumor. Cause of death was classified as related to aplastic anemia or its treatment (bleeding or infection), secondary to late complications, unrelated to aplastic anemia, or unknown. Response to immunosuppressive therapy was defined as reaching complete independence from transfusions. Relapse was defined as dependence on transfusions after 3 months of independence from transfusions.

Statistical Analysis

Group differences were analyzed by using the Kruskal-Wallis test for continuous variables and the Fisher exact test for categorical variables. Survival probabilities were calculated by using the Kaplan-Meier estimator. Time at risk started at the date of first treatment and ended at the date of an event (response to immunosuppressive therapy, relapse, complication, or death) or the date of last known vital status, whichever came first. We calculated 95% CIs of survival probabilities according to the method of Rothman and Boice. Variables significantly associated with the risk for death were assessed by univariate and multivariate analysis. A two-sided log-rank test was used for comparisons. Because patients were treated at 56 centers, the possibility that center-specific differences in supportive care and patient selection would bias the results cannot be excluded. Therefore, univariate survival comparisons between age groups were made by using the log-rank test stratified on center. Sixty-eight percent of patients were treated at 6 centers (39 to 191 patients per center); 32% of patients were treated at 50 centers (1 to 24 patients per center). Grouping the latter patients led to 7 centers that were used for stratification. Proportional hazards regression analysis was used to assess the effect of known risk factors on survival. Variables considered were sex, age group, disease severity (reflected by neutrophil count at diagnosis), type of treatment (antilymphocyte globulin, cyclosporine, or both), and calendar year (1974 to 1979, 1980 to 1989, or 1990 to 1997). A backward stepwise procedure was used to eliminate nonsignificant variables (cut-off value, P > 0.2).

To adjust for the inherently increased risk for death with older age, the number of deaths observed after immunosuppressive treatment for aplastic anemia was compared with the expected number of deaths in a general European population matched for sex and age. The standardized mortality ratio (observed deaths/expected deaths) was calculated for each year after treatment. The 95% CI of the standardized mortality ratio was calculated by assuming a Poisson distribution of the number of observed deaths. The changes in risk for events over time were computed for the whole study sample as well as for the three groups separately. Statistical analysis was performed by using the SPSS statistical program (SPSS for Windows, release 6.1, SPSS, Inc., Chicago, Illinois).

Role of the Funding Source

The funding source had no role in the collection, analysis, or interpretation of the data or in the decision to submit the paper for publication.

Results

Patients

Demographic and disease characteristics of all 810 patients are listed in Table 1. Significant differences were seen among age groups. More female patients were 60 years of age or older, and more men were in the reference group (P < 0.001). Fewer cases of viral-associated aplastic anemia were seen in patients 50 to 59 years of age, and no cases were seen in those 60 years of age or older (P = 0.008). More patients 60 years of age or older received cyclosporine alone, and more patients in the reference group received antilymphocyte globulin (P < 0.001). The proportion of older patients increased continuously over time: Until 1979, 13% of the patients were 50 years of age or older; this proportion increased to 27% from 1980 to 1989 and to 38% since 1990. Hence, the median follow-up
Table 1. Characteristics of 810 Patients with Aplastic Anemia

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All Patients (n = 810)</th>
<th>Patients &gt;60 Years of Age (n = 127)</th>
<th>Patients 50 to 59 Years of Age (n = 115)</th>
<th>Patients 20 to 49 Years of Age (n = 568)*</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years), y</td>
<td>56 (20-89)</td>
<td>67 (60-89)</td>
<td>54 (50-59)</td>
<td>30 (20-49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Males, n (%)</td>
<td>439 (54)</td>
<td>52 (41)</td>
<td>54 (47)</td>
<td>312 (56)</td>
<td></td>
</tr>
<tr>
<td>Neutrophil count at diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 x 10^9 cells/μL</td>
<td>206 (26)</td>
<td>22 (17)</td>
<td>25 (23)</td>
<td>159 (28)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0.2 - 0.5 x 10^9 cells/μL</td>
<td>293 (36)</td>
<td>56 (44)</td>
<td>44 (38)</td>
<td>150 (34)</td>
<td></td>
</tr>
<tr>
<td>0.5 - 1.0 x 10^9 cells/μL</td>
<td>268 (33)</td>
<td>39 (31)</td>
<td>38 (33)</td>
<td>159 (34)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>43 (5)</td>
<td>10 (8)</td>
<td>8 (7)</td>
<td>25 (4)</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Cause of aplastic anemia, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Idiopathic</td>
<td>551 (68)</td>
<td>93 (74)</td>
<td>84 (73)</td>
<td>374 (66)</td>
<td></td>
</tr>
<tr>
<td>Viral</td>
<td>37 (5)</td>
<td>0 (0)</td>
<td>3 (3)</td>
<td>24 (4)</td>
<td></td>
</tr>
<tr>
<td>Drugs</td>
<td>116 (14)</td>
<td>17 (13)</td>
<td>21 (18)</td>
<td>78 (13)</td>
<td>0.0003</td>
</tr>
<tr>
<td>Other</td>
<td>106 (13)</td>
<td>17 (13)</td>
<td>7 (6)</td>
<td>82 (14)</td>
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<td>Initial treatment, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Antithymocyte globulin</td>
<td>547 (68)</td>
<td>75 (59)</td>
<td>76 (66)</td>
<td>396 (70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antithymocyte globulin + cyclosporine</td>
<td>324 (25)</td>
<td>30 (24)</td>
<td>27 (22)</td>
<td>147 (26)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>55 (7)</td>
<td>22 (17)</td>
<td>12 (10)</td>
<td>25 (4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Treatment courses, n (%)</td>
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<tr>
<td>One</td>
<td>623 (77)</td>
<td>102 (80)</td>
<td>85 (74)</td>
<td>436 (77)</td>
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<tr>
<td>Two</td>
<td>142 (17)</td>
<td>19 (15)</td>
<td>23 (20)</td>
<td>89 (16)</td>
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<tr>
<td>Three</td>
<td>34 (5)</td>
<td>6 (5)</td>
<td>4 (3)</td>
<td>26 (5)</td>
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<tr>
<td>More than three</td>
<td>13 (2)</td>
<td>2 (2)</td>
<td>3 (3)</td>
<td>8 (1)</td>
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<td>Years of treatment, n (%)</td>
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<tr>
<td>1971 - 1976</td>
<td>62 (8)</td>
<td>3 (2)</td>
<td>5 (4)</td>
<td>54 (10)</td>
<td></td>
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<tr>
<td>1976 - 1979</td>
<td>480 (59)</td>
<td>61 (48)</td>
<td>70 (61)</td>
<td>496 (86)</td>
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<tr>
<td>1990 - 1997</td>
<td>208 (26)</td>
<td>63 (50)</td>
<td>40 (35)</td>
<td>165 (29)</td>
<td>&lt;0.001</td>
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<td>Calendar year and initial treatment</td>
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<tr>
<td>1974 - 1976</td>
<td>62</td>
<td>3</td>
<td>2</td>
<td>54</td>
<td></td>
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<tr>
<td>1980 - 1989</td>
<td>403 (84)</td>
<td>50 (52)</td>
<td>57 (81)</td>
<td>296 (85)</td>
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<tr>
<td>Antithymocyte globulin, n (%)</td>
<td>66 (14)</td>
<td>5 (15)</td>
<td>10 (14)</td>
<td>47 (13)</td>
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<tr>
<td>Antithymocyte globulin + cyclosporine, n (%)</td>
<td>11 (2)</td>
<td>2 (3)</td>
<td>3 (4)</td>
<td>5 (2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cyclosporine, n (%)</td>
<td>138 (5)</td>
<td>21 (33)</td>
<td>17 (25)</td>
<td>102 (61)</td>
<td></td>
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<tr>
<td>Antithymocyte globulin + cyclosporine, n (%)</td>
<td>49 (18)</td>
<td>70 (123)</td>
<td>5 (22)</td>
<td>19 (11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Follow-up (range), mo</td>
<td>43 (1-238)</td>
<td>31 (1-127)</td>
<td>47 (1-185)</td>
<td>45 (1-238)</td>
<td>&lt;0.001</td>
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</tbody>
</table>

* Number of mortal cases

was 47 months for surviving patients 50 to 59 years of age and 31 months for patients 60 years of age or older compared with 44 months in reference patients (P < 0.001). Severity of disease, as reflected by neutrophil counts at diagnosis and number of courses of immunosuppressive treatment, did not significantly differ among the groups.

Multiple interrelations were found among calendar year, type of immunosuppressive therapy, neutrophil count at diagnosis, and age. Calendar year was significantly associated with type of treatment (Table 1). Before 1980, all patients received antithymocyte globulin alone; since 1990, 51% patients received antithymocyte globulin and cyclosporine, 31% received antithymocyte globulin alone, and 18% received cyclosporine alone (P < 0.001). Since 1990, however, the type of immunosuppressive therapy was not equally distributed among the age groups (Table 1). Patients 60 years of age or older more often received cyclosporine alone (32%), whereas most patients in the reference group received a combination of antithymocyte globulin and cyclosporine (61%) (P < 0.001). Finally, disease severity was associated with age and calendar year.

Before 1980, neutrophil counts less than 0.2 x 10^9 cells/L were encountered in 44% of patients in the reference group. 20% of patients 50 to 59 years of age, and none of the patients 60 years of age or older. Since 1990, low neutrophil counts were observed in 19% of patients in the reference group. 15% of patients 50 to 59 years of age, and 55% of patients 60 years of age or older.

Survival

At the time of last follow-up, 552 of 810 patients (68%) were alive and 258 (32%) had died (survival rate at 5 years, 67% [95% CI, 65% to 69%]). Survival was influenced by age: The older the patients were at diagnosis, the lower the survival rate (Table 2). Survival also improved over time; the 5-year survival rate was 52% (CI, 39% to 64%) in patients treated before 1980 compared with 65% (CI, 60% to 69%) in patients treated from 1980 to 1989 and 73% (CI, 66% to 88%) in patients treated since 1990 (P < 0.001). This improvement was most pronounced in the reference group (P < 0.001) and was not statistically significant in patients 60 years of age or older (Table 2).
Table 2. Five-Year Survival by Univariate Analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>All Patients</th>
<th>Patients 50-59 Years of Age</th>
<th>Patients 50-59 Years of Age</th>
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<tr>
<td></td>
<td>Survival Rate (95% CI)</td>
<td>Survival Rate (95% CI)</td>
<td>Survival Rate (95% CI)</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Overall survival</td>
<td>57 (63-70)</td>
<td>50 (39-50)</td>
<td>57 (46-68)</td>
</tr>
<tr>
<td>Severity of disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil count &lt; 0.2 x 10^9 cells/L</td>
<td>45 (37-52)</td>
<td>21 (6-42)</td>
<td>40 (19-59)</td>
</tr>
<tr>
<td>&lt; 0.5 x 10^9 cells/L</td>
<td>69 (62-74)</td>
<td>40 (14-74)</td>
<td>52 (35-67)</td>
</tr>
<tr>
<td>Neutrophil count &gt; 0.5 x 10^9 cells/L</td>
<td>70 (75-81)</td>
<td>54 (34-70)</td>
<td>72 (53-83)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>57 (61-71)</td>
<td>45 (22-62)</td>
<td>56 (40-60)</td>
</tr>
<tr>
<td>Female</td>
<td>60 (61-71)</td>
<td>53 (39-65)</td>
<td>57 (42-70)</td>
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<tr>
<td>Immunosuppressive therapy</td>
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<tr>
<td>Antithymocyte globulin + cyclosporine</td>
<td>72 (63-80)</td>
<td>47 (24-67)</td>
<td>56 (32-74)</td>
</tr>
<tr>
<td>Antithymocyte globulin</td>
<td>64 (50-68)</td>
<td>50 (37-64)</td>
<td>57 (45-68)</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>67 (71-76)</td>
<td>47 (24-67)</td>
<td>60 (40-70)</td>
</tr>
<tr>
<td>Year of treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1976-1979</td>
<td>52 (39-64)</td>
<td>49 (26-62)</td>
<td>55 (43-66)</td>
</tr>
<tr>
<td>1980-1983</td>
<td>65 (60-69)</td>
<td>50 (37-64)</td>
<td>60 (40-70)</td>
</tr>
<tr>
<td>1990-1997</td>
<td>73 (63-81)</td>
<td>60 (40-70)</td>
<td>60 (40-70)</td>
</tr>
</tbody>
</table>

* Reference group
† Two-year rate not adjusted on death rate
‡ Less than 2 patients were at risk.

Causes of Death

Two hundred fifty-eight patients (32%) died. The mortality rate was 39% (45 of 115) for patients 50 to 59 years of age and 43% (55 of 127) for patients 60 years of age or older compared with 22% (158 of 684) for patients in the reference group. The main cause of death was directly related to aplastic anemia: Bleeding or infection occurred in 205 of 258 patients (79%). Thirty of 258 patients (12%) died of late complications, and 23 of 258 patients (9%) died of causes unrelated to aplastic anemia or its treatment. In all three age groups, bleeding or infection was the main cause of death (Table 3). The excess of mortality in older patients was due to aplasia-related deaths (mortality rate: 32% in patients 50 to 59 years of age; and 43% in patients 60 years of age and older: P < 0.02) and to causes unrelated to the disease or its treatment (mortality rate: 13% in patients 50 to 59 years of age, 4% in patients 60 years of age, and 2% in patients 20 to 49 years of age: P = 0.0019) but not to late complications (4% for all three age groups; P > 0.2).

Response to Immunosuppressive Therapy, Relapse of Aplastic Anemia, and Late Complications

Of 810 patients, 721 (89%) were evaluable for hematologic response. Of these patients, 379 (53%) responded to therapy and reached independence from transfusion. Response to therapy was slow: The median time to independence from transfusion was 6 months. Probability of response to immunosuppressive therapy was 11% (CI: 9% to 14%) at 1 month, 38% (CI: 33% to 42%) at 3 months, 48% (CI: 44% to 52%) at 6 months, and 62% (CI: 58% to 65%) at 12 months (Table 3). In univariate analysis, the probability of response was slightly lower in patients 60 years of age or older than in patients in the reference group (P = 0.0287); this difference was not significant in multivariate analysis (P > 0.2).

Relapse occurred in 66 of the 379 patients who reached independence from transfusion. The cumulative probability of relapse was 21% (CI: 17% to 26%) at 5 years (Table 3). The three age groups did not differ in this regard (P > 0.2).

Seventy of the 810 patients (9%) developed a late complication. Twenty-five patients had a myelodysplastic syndrome or leukemia, 28 patients had a paroxysmal nocturnal hemoglobinuria, 14 patients had a solid tumor, 2 patients had paroxysmal nocturnal hemoglobinuria and myelodysplastic syndrome, and 1 patient had solid tumor and myelodysplastic syndrome (Table 3). The cumulative probability of a late clonal disorder at 10 years was 20% (CI: 15% to 27%). The three age groups did not differ for rate of late complications (P > 0.2).

Risk Factor Analysis

Factors influencing survival, as assessed by univariate analysis, are summarized in Table 2. In any age group, a neutrophil count less than 0.2 x 10^9 cells/L was associated with a lower survival rate (P = 0.01). The effect of age on survival persisted for the degree of neutropenia (Table 2). Significant differences for calendar year of treatment were seen in patients 50 to 59 years of age (P = 0.03) and in the reference group (P < 0.001). In patients 60 years of age or older, survival did not differ significantly by year of treatment (P > 0.2). In the refer-
ence group only, combination therapy with antilymphocytic globulin and cyclosporine was associated with increased survival compared with antilymphocytic globulin therapy alone or cyclosporine therapy alone (P = 0.0018). Sex was not associated with survival. Data analyzed with and without adjustment on center did not differ, excluding center-specific differences of the results (Table 2).

Multivariate analysis confirmed an increased relative risk for death in patients 50 to 59 years of age (relative risk, 1.80 [CI, 1.29 to 2.52]; P < 0.001) and those 60 years of age or older (relative risk, 2.57 [CI, 1.87 to 3.53]; P < 0.001) compared with patients in the reference group. Other significant covariates were a neutrophil count less than 0.2 x 10^9 cells/L (relative risk, 3.41 [CI, 2.50 to 4.67]; P < 0.001), a neutrophil count of 0.20 to 0.50 x 10^9 cells/L (relative risk, 1.47 [CI, 1.06 to 2.04]; P = 0.019), and treatment before 1990 (relative risk, 1.87 [CI, 1.37 to 2.50]; P < 0.001). The relative risk for death was not increased according to sex, type of treatment, or number of courses of immunosuppressive therapy. No significant interactions were seen among age group, neutrophil count at diagnosis, and calendar year. When calendar year was excluded as a risk factor, the risk for death was higher in patients who received antilymphocytic globulin alone (relative risk, 1.52 [CI, 1.08 to 2.15]; P = 0.017) than in those who received antilymphocytic globulin and cyclosporine (baseline relative risk, 1.0). The risk for death among patients treated with cyclosporine alone was not significantly increased (relative risk, 1.33 [CI, 0.72 to 2.44]; P > 0.2). The 5-year survival curves by age group adjusted for neutrophil count and calendar year of treatment are shown in the Figure.

Comparison of Patients with the General Population
The standardized mortality ratio observed in all patients relative to that expected in the general population was 18.64 (CI, 16 to 21; P < 0.001) for a total of 258 observed deaths. The standardized mortality ratio was highest in the first year (60.49 [CI, 52 to 70]); it decreased progressively during the first decade and remained between 5 and 10 in long-

Table 3. Causes of Death, Response to Immunosuppressive Therapy, Relapse, and Late Complications

<table>
<thead>
<tr>
<th>Event</th>
<th>All Patients (n = 812)</th>
<th>Patients &gt;50 Years of Age (n = 127)</th>
<th>Patients 50 to 59 Years of Age (n = 115)</th>
<th>Patients 20 to 49 Years of Age (n = 568)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death</td>
<td>228 (32)</td>
<td>55 (43)</td>
<td>45 (39)</td>
<td>158 (28)</td>
</tr>
<tr>
<td>Causes of death</td>
<td>205</td>
<td>40</td>
<td>37</td>
<td>128</td>
</tr>
<tr>
<td>Blinding or infection</td>
<td>20</td>
<td>5</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Late complications</td>
<td>23</td>
<td>10</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Relapse in patients who responded to immunosuppressive therapy</td>
<td>655/759 (86)</td>
<td>662/158 (42)</td>
<td>121/153 (76)</td>
<td>512/596 (86)</td>
</tr>
</tbody>
</table>

*Reference group

P < 0.0003 between patients older than 60 years of age and patients 20 to 49 years of age.
term survivors (>10 years of follow-up). The standardized mortality ratio decreased with increasing age (Table 4). It was highest in the reference group (33.69 [CI, 29 to 39]), intermediate in patients 50 to 59 years of age (15.75 [CI, 11 to 20]), and lowest in patients 60 years of age or older (9.05 [CI, 6.7 to 12]). In all three age groups, a similar decrease in the standardized mortality ratio was observed over time from the start of immunosuppressive therapy.

Because the year in which treatment was started influenced survival (Table 2), the standardized mortality ratio was also analyzed by calendar period and by calendar period and age group. Standardized mortality ratios were 28.37 (CI, 20 to 40) in 1974 to 1979, 18.63 (CI, 16 to 21.6) in 1980 to 1989, and 14.79 (CI, 10.9 to 19.6) in 1990 to 1997. In 1980 to 1989, standardized mortality ratios were 36.17 (CI, 30 to 43), 13.38 (CI, 9.1 to 19), and 7.64 (CI, 5.1 to 11) in patients aged 20 to 49 years, 50 to 59 years, and 60 years or older, respectively. In 1990 to 1997, the standardized mortality ratios were 24.05 (CI, 15.6 to 40) in patients aged 20 to 49 years, 17.04 (CI, 8.1 to 31) in patients aged 50 to 59 years, and 11.31 (CI, 7.2 to 17) in patients aged 60 years or older.

**Discussion**

This retrospective, multicenter study, based on data from 810 patients, shows that response of aplastic anemia after immunosuppressive therapy is independent of patient age at time of treatment. However, survival differs by age; rates were 57% in patients 50 to 59 years of age and 50% in patients 60 years of age or older compared with 72% in patients 20 to 49 years of age. The relative risk for death was 1.83 in patients 50 to 59 years of age and 2.52 in patients 60 years of age or older. At any age, deaths were mainly due to the primary disease (bleeding and infection). In patients whose disease responded to immunosuppressive therapy, the relapse rate and the probability of late complications, such as the myelodysplastic syndrome, leukemia, paroxysmal nocturnal hemoglobinuria, or solid tumors, did not differ by age. The probability of reaching independence from transfusion at 12 months was greater than 50% for patients at any age.

The effect of age on survival is mainly disease related and is most pronounced in the first year after treatment. Thirty-two percent of patients 50 years of age or older died of hemorrhage or infectious complications compared with 23% of patients in the reference group. Severity of disease was not more pronounced in older age groups, but patients 50 years of age or older are less likely to tolerate the consequences of bleeding and infection, which complicate the management of older patients undergoing immunosuppressive therapy. To evaluate the contribution of expected age-related deaths to the decrease of survival after immunosuppressive

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**Table 4. Standardized Mortality Ratio for Premature Death, Adjusted for Age and Sex, in Patients Treated for Aplastic Anemia with Immunosuppressive Therapy**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients ≥50 Years of Age (n = 127)</th>
<th>Patients 50 to 59 Years of Age (n = 115)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Observed Deaths</td>
</tr>
<tr>
<td>Years after treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>127</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>63</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>5-10</td>
<td>29</td>
<td>3</td>
</tr>
<tr>
<td>&gt;10</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Total patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>52</td>
<td>24</td>
</tr>
<tr>
<td>Women</td>
<td>75</td>
<td>31</td>
</tr>
<tr>
<td>All patients</td>
<td>127</td>
<td>55</td>
</tr>
</tbody>
</table>

*Reference group.*
therapy in older patients, the mortality rate was compared with that of a general population matched for sex and age. Compared with the general population, the risk for death was elevated at all times and in all age groups; the highest ratios were observed in the first year after treatment. Compared with younger patients, older patients had a lower standardized mortality ratio because of the inherent higher risk for death in elderly healthy persons. Therefore, to some extent at least, the decrease in survival among older patients is due to the deaths that can be expected in older persons in a general population.

As for bone marrow transplantation (4, 7, 23), outcome after immunosuppressive therapy has improved over the years from a 5-year survival rate of 52% before 1980 to a rate of 73% since 1990. This improvement in outcome is more pronounced in young patients and is marginal in patients 50 years of age or older. We assume that changes in type of immunosuppressive therapy and patient selection partially explain the strong year-of-treatment effect. Since 1990, overall, the use of combined immunosuppressive therapy with antithymocyte globulin and cyclosporine has increased, whereas most older patients received cyclosporine alone. In a randomized study, combined treatment was shown to be more effective than antithymocyte globulin alone (10), but no difference was found between antithymocyte globulin alone or cyclosporine alone (24). Patient selection has changed as well: Before 1980, younger patients with very low neutrophil counts were usually treated; since 1990, more older patients with less severe disease received immunosuppressive therapy. Apparently, with increasing experience, centers have progressively increased their age limit for immunosuppressive therapy and have begun to institute treatment earlier in the course of disease. Our results contrast with those of a recent single-center study from the Seattle Bone Marrow Transplant Team (25) in which the year of treatment did not affect survival in 227 patients treated with immunosuppressive therapy. In that series, however, cyclosporine was not included in the antithymocyte globulin-based treatment regimen, and the observation time ended in 1991.

Previous studies have reported severity of disease as the main factor affecting outcome in patients treated with immunosuppressive therapy (12, 25). We confirmed the worse prognosis of patients with very severe disease; the relative risk for poor outcome was 3.36 in patients with neutrophil counts less than $0.2 \times 10^9$ cells/L and 1.47 in those with counts between 0.2 and $0.5 \times 10^9$ cells/L compared with patients with higher neutrophil counts. Data on the effect of age on outcome of patients treated with immunosuppressive therapy are sparse. They are mostly focused on children or younger adults (9, 25, 26), and patients 50 years of age or older have not been specifically evaluated. We show that in all age groups, survival is worst in patients with very low neutrophil counts at diagnosis. However, in patients 60 years of age or older, higher neutrophil counts are less clearly associated with outcome, suggesting that increasing age partially eliminates the effect of severity of the disease. No effect of age or disease severity on the development of late complications was seen, despite the fact that older patients are expected to develop more malignant disorders and despite the known increased risk for late clonal disorder among patients with aplastic anemia (17, 19, 27-31).

Some limitations apply to our study, largely because of the retrospective, multicenter character of the analysis. It covered a 23-year period and included 56 participating centers; during this time, changes occurred in supportive care, and concomitant drugs, such as androgens, corticosteroids, or growth factors, have often been administered with immunosuppressive therapy. Different brands and doses of antithymocyte globulin and cyclosporine were administered by the centers. Although the repercussions of these variables on the outcome of patients with aplastic anemia remain largely undetermined, these factors may affect the results of therapy (3, 32-34). Furthermore, potential confounding due to center effect was assessed in part by stratifying the survival comparisons on large centers between age groups (after the small centers were grouped). Finally, younger patients are more often selected for bone marrow transplantation when a donor is available. This selection may bias the reference group and affect our data.

Despite these limitations, immunosuppression is a feasible and effective treatment in older patients,
and there is no reason to withhold treatment in older patients with aplastic anaemia solely because of their age. The decision to treat older patients should depend on the medical condition of the individual patient rather than a fixed age limit.

Appendix

This study was a collaboration of the physicians of 56 centers in the European Group for Blood and Marrow Transplantation. Austria: University Hospital, Innsbruck; University Hospital, Vienna. Belgium: Cliniques Universitaires St. Luc, Brussels. France: Hôpital A. Michallon, Grenoble; Hôpital E. Herriot, Lyon; Hôpital St. Louis, Hôpital Pitié-Salpêtrière, and Hôtel-Dieu, Paris. Germany: Universität Ulm, Ulm. Greece: Evangelismos Hospital, Athens. Ireland: St. James Hospital Trinity College, Dublin. Italy: Ospedale di Torretto di Ancora, Ancora; Università degli Studi di Bari, Bari; Ospedale Bergamo, Bergamo; Hospital San Osvaldo, Bologna; Ospedale San Maurizio, Bologna; Spedali Civili-Brescia, Brescia; Ospedale Oncologico, Cagliari; Ospedale di Ceglie, Firenze; Ospedale di San Martino, Genoa; Institute G. Gaslini, Genoa; Ospedale San Gerardo, Monza; Ospedale di Niagardi, University of Milan, and Instituto Scientifico H.S. Raffaele, Milan; University of Naples, Naples; Ospedale San Francesco, Nauoro; Ospedale V. Cerrulo-USSL 60, Palermo; Poliomicion San Matren, Pavia; Ospedale Civile, Pescara; University of Pisa, Pisa; Azienda Ospedaliera Bianchi-Melancon-Morbidii, Reggio Calabria; Università Cattolica S. Cuore and Università degli Studi La Sapienza, Rome; Hospital Casa Sollievo della Sofferenza, San Giovanni Rotondo; University Hospital of Turin and Ospedale Regina Murgheria, Turin; Udine University Hospital, Udine; University of Verona, Verona; and S. Bartolo Hospital, Vicenzo. The Netherlands: Dr. Daniel D. Hoed Cancer Center, Rotterdam. Poland: K. Dulska Hospital, Institute of Immunology and Experimental Therapy, Wrocław. Portugal: R. Prof. Lima Busto, Lisbon. Saudi Arabia: King Faisal Specialist Hospital, Riyadh. South Africa: Wynberg Hospital, Wynberg. Spain: Postgraduate School of Haematology, Barcelona; University Hospital Universitario Marques de Valdecilla, Santander; Sweden: Huddinge Hospital, Huddinge; University Hospital, Lund. Switzerland: Kantonsspital, Basel; University Hospital, Zürich. Turkey: Marmara Üniversitesi Hastanesi, İstanbul. United Kingdom: Royal Infirmary, Edinburgh; Hammersmith Hospital, University College London Hospital, and St. George’s Hospital, London.

From Kantonsspital Basel, Basel, Switzerland; Hôpital St. Louis, Paris; France: Centre François Bucchini, Caen, France; St. George’s Hospital, London, United Kingdom; Universität Ulm, Ulm, Germany; St. James Hospital Trinity College, Dublin, Ireland; Southmead Health Services, Bristol, United Kingdom; Huddinge Hospital, Huddinge, Sweden; Postgraduate School of Haematology, Faroer. Spain: Hospital San Gerardo, Monza, Italy; and Ospedale San Martino, Genoa, Italy.

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References


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Special report

Outcome of 5651 hematopoietic stem cell transplants for hematological malignancies carried out in Europe in 1993: a reliability study of the registry

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Summary:

Outcome results of observational databases are frequently criticized as relying on incomplete information from incomplete patient populations. Few data are available to dispute these arguments of selection bias. The European Group for Blood and Marrow transplantation (EBMT) decided to address this question by evaluating the hematopoietic stem cell transplants performed in 1993. A comprehensive survey was launched in an effort to collect informations on all transplants for hematological malignancies performed throughout Europe during the year 1993. The main goals of this effort were to compare the group of spontaneously reported patients with the group of retrospectively solicited patients, and to give an accurate estimate of the outcome of all patients. For the year 1993, the annual EBMT activity survey indicated 6336 transplants performed for hematological malignancies in Europe. A total of 5651 transplants could be analyzed; 2595 were reported spontaneously by the teams (group A) and 3056 were retrieved on solicitation (group B). Patients and transplant characteristics for group A and B were very similar for most parameters with a few exceptions. There was no statistical difference for outcome at 3 years between groups A and B: disease-free survival (DFS) was 45 ± 1% and 44 ± 1%, relapse incidence (RI) 41 ± 1% and 42 ± 1%, transplant-related mortality (TRM) 23 ± 1% and 23 ± 1%, and overall survival (OS) 54 ± 1% and 55 ± 1%, respectively, for group A and group B. The real outcome at 3 years for the 5651 patients (group A + group B) transplanted in 1993 was 44 ± 1%, 41 ± 1%, 23 ± 1%, and 54 ± 1%, for DFS, RI, TRM and OS, respectively. The outcome at 3 years by transplant modality, autologous or allogeneic transplants, and by disease categories showed no difference between groups A and B.

Bone Marrow Transplantation (2002) 30, 637–643. doi:10.1038/sj.bmt.1703712

Keywords: hematopoietic stem cell transplantation; observational vs solicited data; actual outcome

A considerable number of scientific observations and analyses have been obtained from data collected retrospectively at centralized registries, which have contributed to the progress of medicine in various ways. In the field of hematopoietic stem cell transplantation, information for the past 20 years has been obtained essentially from two registries, the International Bone Marrow Transplantation Registry (IBMTR)1,2 based in Milwaukee (USA), and the disease-oriented registries of the European Group for Blood and Marrow transplantation (EBMT)3–7 handled by working parties in various locations throughout Europe with centralized management offices in Paris and London. These registries have contributed to the identification of numerous prognostic factors for the treatment of various diseases and to the design of technical improvements for successful transplants. Several prospective randomized studies have been built from and have indeed confirmed many observations first obtained from the registries.

Analyses from registries have been criticized as relying on suboptimal and nonconsecutive data reporting from voluntary teams only, with lack of internal or outside data quality controls. Suspicion has been raised that reported data are biased and have not in fact been an objective reflection of the reality.

The EBMT has two independent data collection procedures. The first, the EBMT activity survey has provided information on the total number of patients transplanted in Europe by each team, year by year, since 1990, according to diagnosis, donor type and stem cell source. The second has consisted of collecting detailed information on patients, diseases, transplant and outcome for retrospective scientific
analyses. This second procedure has not concerned all transplants and has depended on interest, commitment and possibilities of individual teams, on a regular voluntary basis.

In 1995, a comprehensive survey was launched in an effort to collect standardized information on all transplants for all diseases performed throughout Europe during the year 1993. The main goals of this effort were to compare the group of patients reported spontaneously with the group of those solicited retrospectively and to give an accurate estimate of outcome following autologous or allogeneic hematopoietic stem cell transplantation for hematological malignancies by providing a precise picture of what really occurred to these patients transplanted in Europe during the year 1993.

Patients and methods

Study design

This is a retrospective study which focused on patients transplanted with autologous or allogeneic hematopoietic stem cells in 1993 for hematological malignancies.

The first endpoint was to assess and to compare two separate cohorts of patients: a group of patients whose data were present in the database and were spontaneously reported by the teams on a voluntary basis (group A, spontaneous group) and a group of patients whose data were retrieved on solicitation by the study committee (group B, solicited group). Information on missing data was obtained from the EBMT activity survey 1993.4

The second endpoint was to give the real outcome of patients with hematological malignancies transplanted with autologous or allogeneic hematopoietic stem cells in Europe in 1993.

For the year 1993, the annual EBMT activity survey indicated 6336 hematopoietic transplants performed in Europe for hematological malignancies, consisting of 3557 autologous transplants and 2779 allogeneic transplants.

Of these 6336 transplants, 3172 (50.06%) were reported spontaneously; 3164 transplants (49.94%) were missing. EBMT teams were requested to forward the missing information for all patients not yet reported and at the same time to give an updated follow-up for all patients. Teams were contacted repeatedly by the investigators until delivery of the full data.

Data collection

Data were collected by standardized questionnaire or electronic database system according to the Minimum Essential Data A form which includes information on patient identification, disease characteristics, transplant details on donor type, stem cell source and minimal information on outcome.

Definition of endpoints

Both groups were compared for distribution of patients and disease characteristics including patient age, gender, diagnosis, stage of disease at transplant, time from diagnosis to transplant (days), source of stem cells and total body irradiation (TBI) in the conditioning regimen.

Outcome analysis concentrated on probabilities of survival (OS), transplant-related mortality (TRM), relapse incidence (RI) and disease-free survival (DFS). DFS was defined as survival without evidence of relapse, the event under study being death or relapse. To evaluate the probability of RI, patients dying either from direct toxicity or from any other cause not related to leukemia were censored. TRM was defined as death while in complete remission. Patients were censored at the time of relapse or at the last follow-up.4 Probabilities at 3 years were evaluated first for all patients transplanted in 1993 whatever the type of transplant and disease category, then separately for autologous and allogeneic transplantation whatever the disease category, and finally for autologous and allogeneic transplantation by disease category. For each of these evaluations results were given the population A and B. Outcome between population A and B was compared.

Outcomes for allogeneic transplantation were separated between outcome of genoidentical allogeneic transplants and outcome of other allogeneic transplants comprising syngeneic, other family related and unrelated allogeneic transplants. When the number of patients in a subgroup was too small (below 30), outcome was not analyzed. To avoid false interpretations, we used the two-tailed $P$ values.

Statistical analysis

All analyses were performed with the SPSS computer program (SPSS Inc, Chicago, IL, USA). Values reported for quantitative variables were median and range. Comparisons of the two groups A and B were with the chi-square and the Mann–Whitney $U$-test. DFS, RI, TRM and OS were estimated by the product-limit method.9 The significance of differences between curves was estimated by the log-rank test (Mantel–Cox).

Results

Population

By June 1998, a total of 5945 patient data forms had been collected from 323 teams in Europe (see appendix) corresponding to 93.8% of the 6336 transplants reported in 1993. From these 5945 transplants, 294 second or subsequent transplants were removed for the analysis. The final analysis was restricted to a population of 5651 patients with first transplants: 2595 patients in group A and 3056 patients in group B.

Distribution

The total population (A + B) of 5651 patients consisted of 60% males and 40% females, with a median age of 34 years (range: 1–70); 16% were children (age ≤16 years old). Autologous transplantation was performed for 3171 patients (56%) and allogeneic transplantation for 2480 (44%). Almost half of the patients (44%) were transplanted
in first complete remission (or in first chronic phase for CML). For autologous transplantation, 57% of patients received bone marrow as source of stem cells, 34% peripheral blood and 9% both. For allogeneic transplantation, 99.3% of patients received bone marrow and 0.7% peripheral blood.

Distribution of autologous transplants, allogeneic transplants and disease categories in the total population A + B, group A and group B is shown in Table 1.

Comparison of distribution between group A and group B indicated no statistical difference for age, sex ratio, source of stem cell except for a higher proportion of autologous transplants in group A (59%) than in group B (54%) (P < 0.0001) and more allogeneic transplantation done in first complete remission in group A (59%) than in group B (45%) (P < 0.0001).

Comparison of distribution by disease between group A and group B

Comparison showed that for autologous transplantations performed for hematological malignancies, both groups were similar for most disease characteristics with a few exceptions listed below shown in group A compared to group B:

- In non-Hodgkin's lymphomas: a shorter median time from diagnosis to transplant (320 versus 398 days, P = 0.001), more TBI (63% vs 29%, P < 10^-4); marrow more frequently used as the source of stem cells (50% vs 44%, P < 10^-4).
- In Hodgkin's disease: a lower proportion of children (2% vs 1%, P = 0.01) and less TBI (8% vs 56%, P < 10^-4).
- In multiple myeloma: more patients classified as responding to chemotherapy at the time of transplant (77% vs 65%, P = 0.03) and less TBI (35% vs 76%, P < 10^-4).
- In acute leukemias: more TBI for acute myeloid leukemia (78% vs 52%, P < 10^-4) and for acute lymphocytic leukemia (92% vs 54%, P < 10^-4), and more patients transplanted in first complete remission for acute lymphocytic leukemia (75% vs 51%, P < 10^-4).

Comparison for allogeneic transplants for hematological malignancies indicated no difference for most parameters with a few exceptions listed below, shown in group A compared to group B:

- In acute myeloblastic leukemia: a longer median time from diagnosis to transplantation (202 vs 160 days, P < 10^-4) and a higher proportion of allogeneic identical sibling transplantation (91% vs 82%, P = 0.002).
- In acute lymphocytic leukemia: a higher median age (20 vs 15 years old, P < 10^-4) and fewer children (42% vs 55%, P = 0.003); a shorter median time from diagnosis to transplantation (242 vs 549 days, P = 0.0001); more patients transplanted in first complete remission (64 vs 35%, P < 10^-4), and a higher proportion of identical sibling allogeneic transplants (76% vs 67%, P = 0.02).
- In myelodysplastic syndromes: a higher median age (38 vs 31 years old, P = 0.03); less patients receiving TBI (63% vs 88%, P = 0.003).
- In chronic myeloid leukemia: more patients transplanted in first chronic phase (76% vs 66%, P = 0.005).
- In multiple myeloma: more female donors (56% vs 33%, P = 0.04).

Outcome at 3 years

On comparison of outcome between groups A and B at 3 years, with all patients combined, whatever the type of transplant and the disease category, there was no statistical difference.

Disease categories in first complete remission (or in first chronic phase for CML). For autologous transplantation, 57% of patients received bone marrow as source of stem cells, 34% peripheral blood and 9% both. For allogeneic transplantation, 99.3% of patients received bone marrow and 0.7% peripheral blood.

Distribution of autologous transplants, allogeneic transplants and disease categories in the total population A + B, group A and group B is shown in Table 1.

Comparison of distribution between group A and group B indicated no statistical difference for age, sex ratio, source of stem cell except for a higher proportion of autologous transplants in group A (59%) than in group B (54%) (P < 0.0001) and more allogeneic transplantation done in first complete remission in group A (59%) than in group B (45%) (P < 0.0001).

Comparison of distribution by disease between group A and group B

Comparison showed that for autologous transplantations performed for hematological malignancies, both groups were similar for most disease characteristics with a few exceptions listed below shown in group A compared to group B:

- In non-Hodgkin's lymphomas: a shorter median time from diagnosis to transplant (320 versus 398 days, P = 0.001), more TBI (63% vs 29%, P < 10^-4); marrow more frequently used as the source of stem cells (50% vs 44%, P < 10^-4).
- In Hodgkin's disease: a lower proportion of children (2% vs 1%, P = 0.01) and less TBI (8% vs 56%, P < 10^-4).
- In multiple myeloma: more patients classified as responding to chemotherapy at the time of transplant (77% vs 65%, P = 0.03) and less TBI (35% vs 76%, P < 10^-4).
- In acute leukemias: more TBI for acute myeloid leukemia (78% vs 52%, P < 10^-4) and for acute lymphocytic leukemia (92% vs 54%, P < 10^-4), and more patients transplanted in first complete remission for acute lymphocytic leukemia (75% vs 51%, P < 10^-4).

Comparison for allogeneic transplants for hematological malignancies indicated no difference for most parameters with a few exceptions listed below, shown in group A compared to group B:

- In acute myeloblastic leukemia: a longer median time from diagnosis to transplantation (202 vs 160 days, P < 10^-4) and a higher proportion of allogeneic identical sibling transplantation (91% vs 82%, P = 0.002).
- In acute lymphocytic leukemia: a higher median age (20 vs 15 years old, P < 10^-4) and fewer children (42% vs 55%, P = 0.003); a shorter median time from diagnosis to transplantation (242 vs 549 days, P = 0.0001); more patients transplanted in first complete remission (64 vs 35%, P < 10^-4), and a higher proportion of identical sibling allogeneic transplants (76% vs 67%, P = 0.02).
- In myelodysplastic syndromes: a higher median age (38 vs 31 years old, P = 0.03); less patients receiving TBI (63% vs 88%, P = 0.003).
- In chronic myeloid leukemia: more patients transplanted in first chronic phase (76% vs 66%, P = 0.005).
- In multiple myeloma: more female donors (56% vs 33%, P = 0.04).

Outcome at 3 years

On comparison of outcome between groups A and B at 3 years, with all patients combined, whatever the type of transplant and the disease category, there was no statistical difference.

Disease categories

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
<th>Total population A + B</th>
</tr>
</thead>
<tbody>
<tr>
<td>All transplants</td>
<td>2975</td>
<td>3005</td>
</tr>
<tr>
<td>Autologous transplants</td>
<td>1537</td>
<td>1634</td>
</tr>
<tr>
<td>Allogeneic transplants</td>
<td>1058</td>
<td>1422</td>
</tr>
<tr>
<td>Genodental</td>
<td>831</td>
<td>1099</td>
</tr>
<tr>
<td>Syngenic</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Other family related</td>
<td>86</td>
<td>116</td>
</tr>
<tr>
<td>Unrelated</td>
<td>132</td>
<td>216</td>
</tr>
<tr>
<td>Missing information</td>
<td>11</td>
<td>71</td>
</tr>
</tbody>
</table>

Disease categories

<table>
<thead>
<tr>
<th>Disease categories</th>
<th>Group A</th>
<th>Group B</th>
<th>Total population A + B</th>
</tr>
</thead>
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<tr>
<td>Acute myeloid leukemia</td>
<td>471</td>
<td>713</td>
<td>1184</td>
</tr>
<tr>
<td>Acute lymphoid leukemia</td>
<td>390</td>
<td>497</td>
<td>887</td>
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<tr>
<td>Myelodysplastic syndrome</td>
<td>76</td>
<td>95</td>
<td>171</td>
</tr>
<tr>
<td>Secondary acute leukemia</td>
<td>29</td>
<td>22</td>
<td>51</td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>331</td>
<td>417</td>
<td>748</td>
</tr>
<tr>
<td>Chronic lymphoid leukemia</td>
<td>6</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>814</td>
<td>491</td>
<td>1305</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>259</td>
<td>312</td>
<td>571</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>138</td>
<td>237</td>
<td>475</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>81</td>
<td>159</td>
<td>240</td>
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</table>
Outcome of HSC transplants in 1993

Figure 1 Outcome at 3 years of patients transplanted in Europe in 1993 for hematological malignancies from group A (2595 patients) and group B (3086 patients). Figure (a) shows DFS, (b) RI and (c) TRM. Results show that outcome between the two groups are identical reflecting the actual outcome of all patients (group A + group B).

was 49 ± 1%, 29 ± 1%, 31 ± 1% and 54 ± 1% for DFS, RI, TRM and OS, respectively.

For other autologous transplants DFS was 32 ± 3% and 31 ± 3% (P = 0.97), RI 34 ± 5% and 34 ± 3% (P = 0.84), TRM 51 ± 4% and 52 ± 3% (P = 0.86) and OS 37 ± 3% and 39 ± 3% (P = 0.6) for group A and group B, respectively. The real outcome at 3 years of all other autologous transplants (group A + group B) was 31 ± 2%, 34 ± 3%, 52 ± 2% and 38 ± 2% for DFS, RI, TRM and OS, respectively.

Outcome at 3 years by disease category

Table 2 shows results on DFS, RI, TRM and OS for autologous transplants, Table 3 for genoidentical autologous transplants and Table 4 for other autologous transplants.

Results by disease category and by transplant modality showed no difference for outcome between group A and B.

Discussion

This study was undertaken to test whether the outcome of patients with hematological malignancies transplanted during 1 year, the year 1993, reported spontaneously to the EBMT did indeed reflect the outcome of all patients transplanted during the same year. For this purpose, additional information was retrieved by solicitation. Results showed very similar Figures for both groups. The observation is important, since the large population of patients in this study allows a very high statistical power. This work has provided reassuring information that patients reported to a transplant registry are representative of all patients and are not a selected group.

A second important consequence of this study has been to produce highly reliable indicators of outcome following transplantation in various diseases, taking advantage of an optimal registry with all consecutive data reported, and a minimum follow-up of 5 years. This has been a considerable effort not only for the investigators who were assigned the task of tracking all transplants done in Europe for the year 1993, but also for all EBMT teams.

With the implementation of the new telematic network designed by the EBMT, it is foreseen that complete consecutive automatic data reporting will be routinely achieved yearly by 2002 and a search such as this will no longer be necessary. The effort has been fruitful since, by all indi-
### Table 2
Outcome at 3 years by disease category of patients with hematological malignancies treated by autologous stem cell transplantation performed in 1993 in Europe and comparison between group A and group B. Results show a similar outcome for the two groups

<table>
<thead>
<tr>
<th>Non-Hodgkin's lymphoma</th>
<th>Multiple myeloma</th>
<th>Hodgkin's disease</th>
<th>Acute myeloid leukemia</th>
<th>Acute lymphoid leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>P</strong></td>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
</tr>
<tr>
<td>DFS (%)</td>
<td>45 ± 2</td>
<td>48 ± 3</td>
<td>0.61</td>
<td>31 ± 5</td>
</tr>
<tr>
<td>RIT (%)</td>
<td>48 ± 2</td>
<td>46 ± 3</td>
<td>0.70</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>TRM (%)</td>
<td>11 ± 2</td>
<td>11 ± 2</td>
<td>0.70</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>OS (%)</td>
<td>58 ± 2</td>
<td>60 ± 3</td>
<td>0.33</td>
<td>50 ± 5</td>
</tr>
</tbody>
</table>

### Table 3
Outcome at 3 years by disease category of patients with hematological malignancies following geno-identical allogeneic stem cell transplantation performed in 1993 in Europe and comparison of outcome between group A and group B. Results show a similar outcome for the two groups

<table>
<thead>
<tr>
<th>Acute myeloid leukemia</th>
<th>Acute lymphoid leukemia</th>
<th>Myelo-dysplastic syndrome</th>
<th>Chronic myeloid leukemia</th>
<th>Multiple myeloma</th>
<th>Non-Hodgkin's lymphoma</th>
<th>Aplastic anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>P</strong></td>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>P</strong></td>
<td><strong>A</strong></td>
</tr>
<tr>
<td>DFS (%)</td>
<td>52 ± 3</td>
<td>48 ± 3</td>
<td>0.58</td>
<td>44 ± 2</td>
<td>42 ± 4</td>
<td>0.95</td>
</tr>
<tr>
<td>RIT (%)</td>
<td>31 ± 3</td>
<td>32 ± 3</td>
<td>0.77</td>
<td>35 ± 4</td>
<td>42 ± 4</td>
<td>0.18</td>
</tr>
<tr>
<td>TRM (%)</td>
<td>34 ± 3</td>
<td>29 ± 3</td>
<td>0.62</td>
<td>33 ± 2</td>
<td>47 ± 4</td>
<td>0.77</td>
</tr>
<tr>
<td>OS (%)</td>
<td>56 ± 3</td>
<td>52 ± 3</td>
<td>0.41</td>
<td>50 ± 4</td>
<td>48 ± 4</td>
<td>0.82</td>
</tr>
</tbody>
</table>

### Table 4
Outcome at 3 years by disease category of patients with hematological malignancies following other allogeneic stem cell transplantation (syngeneic, other family related and unrelated) performed in 1993 in Europe and comparison of outcome between group A and group B. Results show a similar outcome for the two groups

<table>
<thead>
<tr>
<th>Acute myeloid leukemia</th>
<th>Acute lymphoid leukemia</th>
<th>Chronic myeloid leukemia</th>
<th>Aplastic anemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
<td><strong>P</strong></td>
<td><strong>A</strong></td>
</tr>
<tr>
<td>DFS (%)</td>
<td>27 ± 10</td>
<td>31 ± 6</td>
<td>0.56</td>
</tr>
<tr>
<td>RIT (%)</td>
<td>52 ± 15</td>
<td>42 ± 8</td>
<td>0.66</td>
</tr>
<tr>
<td>TRM (%)</td>
<td>44 ± 11</td>
<td>45 ± 7</td>
<td>0.7</td>
</tr>
<tr>
<td>OS (%)</td>
<td>32 ± 10</td>
<td>41 ± 6</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Indicators available, including the yearly EBMT caseload reports, the 5945 transplants collected for this study probably represents 93.8% of all transplants done in Europe for hematological malignancies during the year 1993. What happened to these patients provides information of potential importance not only in terms of therapeutic management, but also in terms of epidemiology and public health. Indeed, more than 50% of patients with hematological malignancies treated by hematopoietic stem cell transplantation during the year 1993 have experienced long-term survival.

This study indicates that the numerous scientific analyses that have been carried out to date on EBMT registries, which contain incomplete information, have nonetheless generated reliable information.

While this observation is reassuring, it must however be emphasized that the conclusions are from a retrospective study and that it would not support by any means that non-exhaustive collection of supposedly representative data from selected centres would constitute the bases for future analysis. Electronic data collection aiming to pick up all transplants is the EBMT goal for the beginning of this century.

### Acknowledgements

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References

Increasing utilization of bone marrow transplantation. II. Results of the 1985-1987 survey.

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The International Bone Marrow Transplant Registry conducts periodic surveys to determine activity in the field of allogeneic and syngeneic bone marrow transplantation. Data were reported to the IBMTR by 258 institutions in 41 countries regarding their patients who received bone marrow transplants during the period 1985-1987. To the best of our knowledge, the data represent essentially all bone marrow transplants (exclusive of autologous transplants) performed in the past 3 years. A total of 10,887 patients received bone marrow transplants; 73% were for leukemia, 11% for other malignant diseases, 9% for severe aplastic anemia and related disorders, 3% for immune deficiency diseases, 2% for thalassemia major, and 2% for genetic, metabolic, and several other rare diseases. 161 (62%) of the 258 institutions performed fewer than one transplant per month. More than 50% of the patients were transplanted in 37 institutions. 46% of the world's bone marrow transplants were performed in North America, 42% in Western Europe, 5% in Asia, 3% in Australia and New Zealand, 2% in the Middle East and Africa, 1% in South and Central America, and 1% in Eastern Europe and the USSR. The data reflect continued growth in utilization of allogeneic and syngeneic bone marrow transplantation and quantify the annual increases in the number of patients receiving transplants.

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Severe Aplastic Anaemia: A Prospective Study of the Effect of Androgens or Transplantation on Haematological Recovery and Survival

BRUCE M. CAMITTA
E. DONNALL THOMAS

for the International Aplastic Anaemia Study Group*

In 1959 Shahidi and Diamond published their article on the use of androgens for treatment of marrow aplasia. Two years later the same authors reported haematological improvement in 9 of 17 children with aplastic anaemia who were treated with testosterone (Shahidi and Diamond, 1961). Confirmatory studies, utilizing a variety of anabolic agents, followed (Sanchez-Medal et al., 1969). However, other workers were unable to demonstrate that androgens modified the course of aplastic anaemia (Heyn, Ertel and Tubergen, 1969; Li, Alter and Nathan, 1972; Williams, Lynch and Cartwright, 1973).

These conflicting results probably reflected the small size of many series, use of historical instead of concurrent controls, and failure to assess adequately disease severity. Thus, androgen responsiveness was seen primarily in patients with milder marrow aplasia (Duarte et al., 1972; Lynch et al., 1975). In contrast, patients with severe marrow aplasia fared poorly whether or not androgens were utilized.

In order to determine the efficacy of androgens for treatment of severe aplastic anaemia, a prospective randomized trial was designed. The results indicate that androgens, as utilized in this study, are not effective treatment for severe aplastic anaemia.

STUDY DESIGN

Only patients with newly diagnosed severe aplastic anaemia were eligible for this study (Table 1). The protocol is outlined in Figure 1. Following diagnosis, patients were observed for 10 days to exclude the presence of an underlying illness, to establish baseline haematological values, to detect early

*See appendix for contributing institutions and physicians.

spontaneous improvement and to complete histocompatibility studies. During this time prednisone (10 mg/m²/day) was given to some patients in an attempt to detect underlying leukaemia (Melhorn, Gross and Newman, 1970). Patients with aplasia secondary to nutritional deficiency, malignancy, irradiation, chemotherapy, preleukaemia, myelofibrosis or Fanconi's anaemia, and patients with other life-threatening diseases, were excluded from the study.

<table>
<thead>
<tr>
<th>Table 1. Criteria for severe aplastic anaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral blood</strong> (two or more of the following):</td>
</tr>
<tr>
<td>Granulocytes</td>
</tr>
<tr>
<td>Platelets</td>
</tr>
<tr>
<td>Anaemia with reticulocytes</td>
</tr>
</tbody>
</table>

**Bone marrow:**
- Severely hypocellular (< 25% of normal) or
- Moderately hypocellular (25-50% of normal) with > 70% of remaining cells being non-haematopoietic

On day 10, patients with persistent severe aplasia were assigned to treatments after obtaining informed consent. Patients without matched siblings were randomized to receive no, oral (oxymetholone, 3 to 5 mg/kg/day) or intramuscular (nandrolone decanoate, 3 to 5 mg/kg/week) androgen. Some physicians were unwilling to randomize patients to no androgens. Their patients were randomized to oral versus intramuscular androgens, resulting in fewer patients on the no androgen treatment as compared to the two androgen regimens.

![Figure 1. Therapeutic guidelines for Aplastic Anaemia Study.](image)

Prednisone (10 mg/m²/day) could be continued at the discretion of the patient's physician. Antibiotics and transfusion support were administered as needed. The initial regimen was continued for at least four months. If no response had occurred, patients could be re-randomized between the remaining regimens. Usually they remained on their initial treatment.

Patients with histocompatible siblings were scheduled for marrow transplantation (to begin on day 21 or as soon thereafter as possible). Details of transplantation procedures have been published previously (Camitta et al, 1976).
Complete response was defined as the return of all haematological values to normal. Partial response meant improvement so that the patient no longer qualified for severe status and no longer required transfusions.

RESULTS

Between July 1, 1974, and June 30, 1977, 111 patients were entered into this study. Treatment groups were comparable for characteristics thought to have prognostic significance in aplastic anaemia (Table 2) except for a slight difference in sex ratio of the no androgen patients. During the 10 d observation period, six patients died and four improved. One of the latter subsequently developed acute lymphocytic leukaemia; a second became severely aplastic and died several months later; the remaining two patients are haematologically normal. These 10 patients were not entered into the study.

Responses and survival of 64 non-transplanted patients are summarized in Table 3 and Figure 2. There are no significant differences in survival times amongst the three regimens. Six living patients have shown no improvement. Twelve patients improved. Responses began after 1 to 13 months with 6 of

<table>
<thead>
<tr>
<th>Androgen</th>
<th>None</th>
<th>PO</th>
<th>IM</th>
<th>Transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>13</td>
<td>27</td>
<td>24</td>
<td>47</td>
</tr>
<tr>
<td>Age (year)</td>
<td>12(3.67)</td>
<td>13(1.77)</td>
<td>12(2.67)</td>
<td>17(2.43)</td>
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<tr>
<td>Male/Female</td>
<td>5/8</td>
<td>19/8</td>
<td>15/9</td>
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<tr>
<td>Aetiology of aplasia:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>13</td>
<td>18</td>
<td>20</td>
<td>36</td>
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<td>Post-hepatitis</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>4</td>
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<td>Drug</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>4</td>
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<tr>
<td>Unrecorded</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
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<tr>
<td>Interval: Symptoms-diagnosis (week)</td>
<td>3(1-58)</td>
<td>3(0-51)</td>
<td>2(0-16)</td>
<td>3(0-17)</td>
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<tr>
<td>Onset:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemorrhagic only</td>
<td>11</td>
<td>15</td>
<td>9</td>
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<td>Infectious only</td>
<td>1</td>
<td>4</td>
<td>2</td>
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<td>Both</td>
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<td>205</td>
<td>187</td>
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<tr>
<td>35-1176</td>
<td>(0-1800)</td>
<td>(0-1500)</td>
<td>(0-5870)</td>
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<tr>
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<td>5000</td>
<td>7243</td>
<td>9600</td>
<td>7000</td>
</tr>
<tr>
<td>900</td>
<td>(18-)</td>
<td>(4000-)</td>
<td>(1000-)</td>
<td></td>
</tr>
<tr>
<td>450000</td>
<td>200000</td>
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<tr>
<td>Reticulocytes ($%$)</td>
<td>0.4</td>
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<td>0.3</td>
<td>0.2</td>
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<tr>
<td>0.1-0.54</td>
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<td>(0.10-1.0)</td>
<td>(0-1.0)</td>
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</tr>
<tr>
<td>Non-myeloid marrow cells ($%$)</td>
<td>68</td>
<td>80</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td>30-98</td>
<td>(20-99)</td>
<td>(35-99)</td>
<td>(30-99)</td>
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Table 3. Status of study patients according to initial therapy (as of 7/1/77). See text for definitions of response

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<th>Treatment</th>
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<th>Total</th>
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<td>2</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>13</td>
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<tr>
<td>Oral</td>
<td>2</td>
<td>5</td>
<td>1</td>
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<tr>
<td>IM</td>
<td>0</td>
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<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Transplantation</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>47</td>
</tr>
</tbody>
</table>

12 responses occurring within two months. There were no differences in time of response amongst the regimens. Reticulocytosis and a decreased red cell transfusion requirement were usually the first sign of improvement. Platelets responded slowly and often incompletely. With one exception responses were progressive, albeit at variable rates. No complete responder was dependent on androgen for maintenance of response. Similarly, almost all partial responders on androgen regimens have been able to discontinue or substantially decrease androgen doses without a haematological relapse.

Forty-six non-transplanted patients died. Time to death was 1 to 33 (median three) months. The causes of death were haemorrhage (23), infection (14) and infection plus haemorrhage (9). There were no differences between regimens in median time to, or cause of, death.

Toxicities of both androgen regimens were mild and in general were overshadowed by complications from the disease per se. Virilization was modest.

---

Figure 2. Life table survival plot of non-transplanted patients according to initial treatment regimen. Squares, triangles and circles indicate duration of follow-up of current survivors.
probably because of the short duration of therapy in most patients. No patient required discontinuation of oxymetholone for hepatocellular dysfunction. Severe haematomas following nandrolone decanoate injection occurred on two occasions but did not require a change of therapy. An abscess developed at the site of injection on only one occasion.

Table 3 and Figure 3 summarize the results in the 48 patients treated with marrow transplantation. Twenty-seven of these patients are alive with complete haematological recovery but four have chronic graft-versus-host disease of mild to moderate severity. The probability of survival following transplantation was slightly greater for children than for adults, but this difference was not significant ($P = 0.10$).

**DISCUSSION**

This study does not support the use of androgens for treatment of severe aplastic anaemia. Time of response, rate of response, percentage responding, median survival time and long-term survival were similar in all three non-transplant regimens. Patient age, sex and initial blood counts did not affect patient survival or response.

It is still possible that a small advantage for androgens might exist. Androgen dependency in aplastic anaemia has been reported (Duarte et al, 1972). However, many of these cases were mild or no details were given regarding severity. Androgen dependency was not demonstrated in this series. Furthermore, the ability of androgens to increase granulopoiesis and thrombopoiesis may be limited. In severe aplastic anaemia, responses of these blood elements are usually delayed (Sanchez-Medal et al, 1969). Since the high early mortality in severe marrow aplasia is due to haemorrhage and/or infection,
more rapidly effective early therapy is necessary. Awaiting androgen responses decreases the zeal with which such alternative treatments are sought.

Survival times of transplanted patients were greater than those of non-transplanted patients \( (P < 0.001) \). If an appropriate donor exists, marrow transplantation is currently the treatment of choice for children and young adults with severe aplastic anaemia. For older patients \( (> 25 \text{ years}) \) in this study, survival times tended to be longer for those transplanted, but not significantly so.

Criteria for severity were devised from data available at the time and confirmed by more recent sophisticated analysis \( (\text{Lynch et al., 1975}) \). The aim was to maximize recovery of all patients while minimizing transplantation of patients who would spontaneously recover \( (\text{Camitta et al., 1975}) \). Clearly the criteria did define a population with a grim prognosis. Preliminary analysis of presenting characteristics has failed to distinguish survivors from non-survivors in our severely affected patients.

Eight individuals with milder aplasia were also followed. In contrast to the severely affected patients, five \( (\text{four of whom received androgens}) \) have shown complete or partial improvement, one remains stable on androgens and two \( (\text{treated with androgens}) \) became severe and died. Mildly affected patients who become severe seem to have the same prognosis as individuals severely affected initially \( (\text{Camitta et al., 1975}) \). Interestingly, one of the complete responders is androgen dependent.

Alternative approaches are needed for patients with severe aplastic anaemia who do not have a compatible sibling marrow donor. Because of the variable and often unpredictable course of aplastic anaemia, it is important that trials be designed prospectively to maximize the probability that meaningful answers are obtained, regardless of the therapy being investigated.

**SUMMARY**

A prospective study was performed \((1)\) to evaluate the role of androgens in treatment of severe aplastic anaemia, and \((2)\) to compare the efficacy of early bone marrow transplantation with more conventional therapy for severe marrow aplasia. Results fail to support a beneficial effect of androgens on the course of patients with severe aplastic anaemia. In contrast, histocompatible bone marrow transplantation significantly improves survival and hematological recovery. Alternative therapies should be carefully evaluated for non-transplantable patients with severe marrow aplasia.
## APPENDIX: CONTRIBUTING INSTITUTIONS AND PHYSICIANS

<table>
<thead>
<tr>
<th>Institution</th>
<th>Principal Investigators</th>
<th>Grant Support</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beth Israel Medical Center, New York City</td>
<td>A. Rausen</td>
<td></td>
</tr>
<tr>
<td>Cardeza Foundation, Philadelphia</td>
<td>A. Fresew</td>
<td>USPHS 1-R-00118</td>
</tr>
<tr>
<td>*Children's Hospital, Boston</td>
<td>D. Nathan, R. Parkman</td>
<td>USPHS AM-65581</td>
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<tr>
<td>Children's Hospital, Los Angeles</td>
<td>S. Siegel</td>
<td></td>
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<tr>
<td>Children's Hospital, Milwaukee</td>
<td>B. Camitta, J. Casper</td>
<td>CA 17700</td>
</tr>
<tr>
<td>Children's Hospital, Philadelphia</td>
<td>S. Friedman, E. Schwartz, C. August</td>
<td>Tommy Fund</td>
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<tr>
<td>Children's Hospital, St Louis</td>
<td>R. Duco, H. Zarkowsky</td>
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<tr>
<td>Cornell Medical Center, New York</td>
<td>A. Moore, D. Miller</td>
<td></td>
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<tr>
<td>Emory University, Atlanta</td>
<td>T. Heffner, J. Keller, A. Ragab</td>
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<tr>
<td>*Hammersmith Hospital, London</td>
<td>E. C. Gordon-Smith</td>
<td>Syntex Pharmaceuticals</td>
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<tr>
<td>Hematology-Oncology Associates P.A., Plainfield</td>
<td>D. Frimmer, C. Leff</td>
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<tr>
<td>Hematology-Oncology Associates, Providence</td>
<td>R. Damico</td>
<td></td>
</tr>
<tr>
<td>James Whitcomb Riley Hospital, Indiana University, Indianapolis</td>
<td>R. Baehner, A. Provisor, R. Weisman, L. Boxer</td>
<td>Riley Memorial Association</td>
</tr>
<tr>
<td>*Johns Hopkins Oncology Center, Baltimore</td>
<td>G. Santos</td>
<td>USPHS 5-PO1-CA-15396</td>
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<tr>
<td>Mary Hitchcock Hospital, Dartmouth</td>
<td>R. Mclntyre</td>
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<tr>
<td>*Memorial Sloan-Kettering, New York</td>
<td>R. O'Reilly, R. Good</td>
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<tr>
<td>Milwaukee County General Hospital</td>
<td>A. Piscotta</td>
<td>USPHS 5-M01-RR-00058</td>
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<tr>
<td>Mount Sinai Hospital, New York City</td>
<td>R. Taub, R. Zalusky</td>
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<tr>
<td>*Naval Medical Center, Bethesda</td>
<td>R. Cahil, D. Pasquale, K. Sell</td>
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<tr>
<td>New Jersey Medical School, Newark</td>
<td>T. Walters</td>
<td></td>
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<td>Pawtucket Memorial Hospital, Providence</td>
<td>M. Baldini</td>
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<td>Peter Bent Brigham Hospital, Boston</td>
<td>J. Rappeport</td>
<td>USPHS 5-M01-RR-00888</td>
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<td>Rhode Island Hospital, Providence</td>
<td>E. Forman, M. Albala</td>
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<td>San Francisco Medical Center</td>
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<td>USPHS CA-17995</td>
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<td>St. Jude Children's Research Hospital, Memphis</td>
<td>R. Strauss, J. Wilimas</td>
<td>ALSAC</td>
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<tr>
<td>Temple University, Philadelphia</td>
<td>J. Day</td>
<td></td>
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<tr>
<td>*Transplant Centres</td>
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ACKNOWLEDGEMENTS

This investigation was supported in part by a grant from Organon, Inc., and by Grant Number CA 18029, awarded by the National Cancer Institute, DHEW. Dr. Thomas is a recipient of Research Career Award AI 03425 from the National Institute of Allergy and Infectious Diseases. We are grateful to Ms. Nancy Flournoy and Mr. Gary Schoch for data management and biostatistical support.

REFERENCES


Registry report

Report from the International Bone Marrow Transplant Registry

Advisory Committee of the International Bone Marrow Transplant Registry

Summary:

The International Bone Marrow Transplant Registry (IBMTR) receives and analyses detailed information contributed by transplant teams at more than 175 institutions worldwide. This collaborative research effort has grown rapidly; there are now more than 8000 cases in the database. This is a summary of the current status of bone marrow transplantation in leukemia and severe aplastic anemia, a brief summary of key findings reported by the IBMTR during the past year, as well as studies planned for the coming year.

The International Bone Marrow Transplant Registry (IBMTR) began in 1970 as a branch of the American College of Surgeons/National Institutes of Health Organ Transplant Registry. Initially, only basic information was collected for transplants performed after 1 January, 1968. In 1973 the Advisory Committee of the IBMTR decided to convert the IBMTR into a research organization. The concept was that by collecting, pooling and analysing detailed data from teams worldwide, progress in the field would be accelerated. That this approach was effective and continues to be perceived as worthwhile is evidenced by the fact that more than 75% of all transplant teams in the world (see Appendix) voluntarily report comprehensive information regarding their consecutive bone marrow transplant patients. It should be noted, however, that many reputable centers do not participate in the IBMTR research program. The IBMTR now has detailed data on more than 8000 patients who received bone marrow transplants and continues to grow both in the number of cases being reported and the number of teams participating in this unique example of international scientific cooperation.

In this report, the IBMTR presents the current status of bone marrow transplantation in leukemia and severe aplastic anemia, a brief summary of several key findings from recent analyses, as well as studies planned for the next year.

Operation of the IBMTR

In brief, data are reported to the Statistical Center of the IBMTR for recipients of allogeneic or syngeneic transplants. Five specialized reporting forms, each containing 450–500 questions, are used to collect information regarding the following: (1) leukemia, lymphoma and other malignant diseases; (2) severe aplastic anemia, Fanconi anemia, pure red cell aplasia, osteopetrosis, paroxysmal nocturnal hemoglobinuria and other related conditions; (3) immunodeficiency diseases; (4) hemoglobinopathies; and (5) genetic and metabolic disorders. Protocols for uniform reporting of data are incorporated into the forms. The reporting forms are updated periodically to eliminate questions for which data are no longer needed and to add questions that take into account advances in the field. Reporting forms are formulated at the Statistical Center by staff members with members of the Advisory Committee and individuals with special expertise acting as consultants. Follow-up forms for each of the five disease categories contain approximately 150 questions, are similarly prepared and are submitted every 6–12 months. To help offset their administrative expenses, teams are reimbursed $100 for an initial report of a transplant, $50 for a report of a second transplant on the same patient and $15 for each follow-up report. Reimbursement is not made until all questions are answered and any ambiguities clarified.

Scientific validity of IBMTR analyses requires that teams report successive cases and that the quality of the reports is high. Several methods are used to ensure consecutive reporting of cases by each team and to evaluate the quality of the data submitted. Teams assign unique patient numbers as patients are transplanted. These numbers are monitored at the Statistical Center. Team leaders are notified when numbers are missing and are required to explain any gaps in the unique patient numbers. Numerous checks are built into the reporting forms to test the quality of the data. Explanations or corrections are requested when inconsistencies are detected.

Several measures also are employed to assure quality management of the data at the Statistical Center. Case reports are registered, coded independently by two different data entry operators and reviewed by a physician to evaluate the quality of the report and to answer questions raised by the data entry operators. When necessary, teams are requested to provide missing information, clarification of ambiguities, explanations for data outside the anticipated range or non-consecutive reporting. The corrected data are then entered into the
Results

Acute lymphoblastic leukemia (ALL)

Life table analyses were performed regarding 1242 consecutive reports of patients with ALL receiving bone marrow transplants from HLA-identical siblings between January, 1978 and December, 1987 to determine the risk of relapse and the probability of leukemia-free survival (Figure 1). The 5-year actuarial probabilities of relapse and leukemia-free survival for these patients are shown in Table I. These data demonstrate the highly significant association between disease status at the time of transplantation and outcome.

Bone marrow transplantation is being used in first remission of ALL for selected patients considered to be at high risk of relapse if treated with chemotherapy. Criteria used by transplant centers to define these patients include: age less than 2 years or older than 15 years, WBC \( \geq 50 \times 10^9/1 \), CNS involvement, B cell or L3 phenotype, or the specific chromosome abnormalities t(4;11), t(8;14) or t(9;22). Analyses were conducted to evaluate outcome following bone marrow transplantation for high-risk ALL. Of 444 patients having these high-risk features at diagnosis, 236 were transplanted while the disease was still in first remission and 208 in second remission. The 4-year probability of relapse was 26±12% (95% confidence interval) for transplants in first remission and 56±11% for transplants in second remission \( (p<0.0001) \). Leukemia-free survival was 45±9% and 22±7%, respectively \( (p<0.0002) \). Also available for study were data on 97 patients having none of the high-risk features at diagnosis and who were transplanted in second remission. Their 4-year probability of relapse was 49±14%, not significantly different from patients with high-risk leukemia at diagnosis transplanted in second remission. Their probability of leukemia-free survival was 36±11%, significantly better \( (p<0.0006) \) than high-risk patients transplanted in second remission.

These data indicate that survival after transplantation for high-risk ALL in first remission was clearly superior to survival after transplantation in second remission. Since a certain number of patients will be cured with chemotherapy alone, and a certain number never achieve second remission after relapse, further studies are needed to determine whether patients with high-risk ALL should receive transplants during first remission or should initially receive chemotherapy, reserving transplantation for patients who relapse.

Acute myelogenous leukemia (AML)

The probability of relapse and leukemia-free survival was determined for 1429 consecutive reports of patients with AML receiving transplants from HLA-identical siblings between 1978 and 1987 (Figure 2). The data at 5 years post-transplant are presented in Table II. The strong association between disease status at transplant and outcome is also shown here.

Table 1 Five-year probability of relapse and leukemia-free survival (± 95% confidence intervals) following HLA-identical sibling bone marrow transplantation for acute lymphoblastic leukemia by disease status at transplant

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease status</th>
<th>n</th>
<th>Relapse %</th>
<th>Univariate p</th>
<th>Leukemia-free survival %</th>
<th>Univariate p</th>
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</thead>
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<tr>
<td>1</td>
<td>1st CR</td>
<td>369</td>
<td>30±8</td>
<td>&lt;0.0001</td>
<td>43±8</td>
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<td>2</td>
<td>≥2nd CR</td>
<td>592</td>
<td>54±7</td>
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<td>26±5</td>
<td>&lt;0.0001</td>
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<td>3</td>
<td>Relapse</td>
<td>281</td>
<td>71±8</td>
<td></td>
<td>13±5</td>
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Table II  Five-year probability of relapse and leukemia-free survival (±95% confidence intervals) following HLA-identical sibling bone marrow transplantation for acute myelogenous leukemia by disease status at transplant

<table>
<thead>
<tr>
<th>Group</th>
<th>Disease status</th>
<th>n</th>
<th>Relapse %</th>
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<td>3</td>
<td>Relapse</td>
<td>321</td>
<td>67±9</td>
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<td>13±7</td>
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Figure 2  Life table analysis among patients with acute myelogenous leukemia showing probability of (a) relapse and (b) leukemia-free survival following bone marrow transplantation from HLA-identical siblings by disease status at transplant.

Chronic myelogenous leukemia (CML)

Disease status at the time of transplant was also found to be significantly correlated with outcome among 1082 patients with CML receiving bone marrow from HLA-identical siblings, 1978-87 (Figure 3). Actuarial 5-year probabilities of relapse and leukemia-free survival are presented in Table III.

Data from 405 consecutive reports of patients with CML who received bone marrow transplants from HLA-identical siblings while in the first chronic phase were analysed to identify factors predicting relapse and survival. The 4-year probability of relapse was 19±8% and of survival, 55±5%. In multivariate analysis the probability of remaining in remission was lower for patients receiving bone marrow depleted of T lymphocytes compared with patients receiving T cell-replete grafts, irrespective of the grade of acute or chronic graft-versus-host disease (GVHD) (relative risk 5.4, p<0.0001). The probability of remaining in remission also was lower for patients who did not have chronic GVHD compared with those who did, irrespective of whether the bone marrow was or was not depleted of T
cells (relative risk 3.1, \( p < 0.01 \)). The probability of survival was lower among patients who developed moderate to severe acute GVHD than in those with no or mild acute GVHD (relative risk 3.7, \( p < 0.0001 \)), and in patients \( > 20 \) years of age than in younger patients (relative risk 2.6, \( p < 0.0002 \)). Duration of disease before transplant was not significantly associated with outcome.

The data indicate that bone marrow transplantation performed in the chronic phase of CML offers some patients a prolonged period of leukemia-free survival. Once transformation into the accelerated or blastic (acute) phase occurs, the results of bone marrow transplantation are significantly poorer. Since transplantation offers the only possibility of cure, the opportunity should not be lost by prolonged delay, especially in younger patients with an HLA-identical sibling.

**Severe aplastic anemia (SAA)**

The actuarial probability of survival among 732 patients with SAA following bone marrow transplantation for HLA-identical siblings between 1978 and 1987 is shown in Figure 4. The 5-year probability of survival was 57\( \pm \)4\% (95\% confidence interval).

Graft failure was analysed in 625 patients receiving allogeneic bone marrow transplants from HLA-identical sibling donors between 1978 and 1986 as treatment for severe aplastic anemia.5 Sixty-eight (11\%) had no or only transient engraftment. Second bone marrow transplants were successful in achieving extended survival in 16 of 27 patients with transient initial engraftment but in none of 10 patients with no sign of engraftment after the first transplant. The major factors associated with a reduced risk of graft failure were use of radiation for pretransplant immunosuppression, and use of cyclosporine rather than methotrexate or T cell depletion of the donor bone marrow for prophylaxis against GVHD. Among 266 patients prepared for transplantation with cyclophosphamide alone, the risk of graft failure was increased in patients who received previous transfusions and reduced in those who received corticosteroids for previous therapy. Neither cell dose nor administration of donor buffy coat cells affected the probability of engraftment. Although use of radiation in conditioning reduced graft failure, survival was not improved. Post-transplant treatment with cyclosporine and avoiding pretransplant blood transfusions were associated with improved survival.

**Risk factors for acute GVHD**

Acute GVHD is another major complication of allogeneic bone marrow transplantation. Data from 2036 consecutive reports of HLA-identical sibling bone marrow transplants for leukemia or aplastic anemia were analysed to identify factors associated with the risk of moderate to severe acute GVHD.6 Patients receiving grafts that were depleted of T cells were excluded from analysis. All patients survived \( > 21 \) days and were thus considered to be at risk of GVHD. The incidence of moderate to severe acute GVHD was 45\( \pm \)2\%, the actuarial risk at 6 months 46\( \pm \)2\%, the case fatality rate 48\% and the mortality rate 22\%. Variables associated with the risk of GVHD are shown in Table IV. The risk of developing acute GVHD was 16\% when none of the adverse risk factors was present. The cumulative probability of moderate to severe acute GVHD with all adverse risk factors added was 87\% and the relative risk was 5.4 times higher than in patients with none of the adverse risk factors.

There was no significant difference in the 44\( \pm \)3\% incidence of GVHD among 1235 patients given methotrexate to prevent or modify GVHD versus the 46\( \pm \)4\% incidence in 710 patients given cyclosporine. Age of the patient at transplant was a significant risk factor for GVHD (relative risk 1.6, \( p < 0.001 \)) ranging from 39\( \pm \)4\% in the lowest age quartile to 52\( \pm \)4\% in the highest. This age effect appeared to be due primarily to the high incidence of GVHD in female--male transplants from previously pregnant or transfused (allo-immune) donors in comparison with similar donors for female recipients (66\( \pm \)9\% versus 39\( \pm \)9\%, respectively.

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**Table III** Five-year probability of relapse and leukemia-free survival following HLA-identical sibling bone marrow transplantation for chronic myelogenous leukemia by disease status at transplant

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<th>Group</th>
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<td>&lt;0.001</td>
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**Figure 4** Life table analysis among patients with severe aplastic anemia showing the probability of survival following bone marrow transplantation from an HLA-identical sibling.
relative risk 2.9, p < 0.0001, Figure 2b). The cohort of 59 older female→male transplants from parous or transfused donors had an incidence of GVHD of 75%, which was the highest seen in this study. Multivariate analysis of all 1818 patients except alloimmune female→male transplants showed that age no longer remained as a significant risk factor for GVHD: 39±3% in younger patients versus 47±3% in older patients (p = 0.30).

Results of this study should be useful in estimating the risk of GVHD in individuals with a single risk factor or a specific profile of risk factors. The data also should prove useful in developing future approaches and in designing randomized clinical trials testing alternative methods of preventing GVHD. For example, if the randomization was not stratified for the prognostic variables identified here, it conceivably would be possible to observe rates of moderate to severe acute GVHD of 16 to >85% by chance alone in either or both arms.

**HLA associations with leukemia**

Although a strong association between H-2 and leukemia in mice has been recognized for more than 20 years, there have been no convincing reports of an association between HLA and leukemia in humans. Previous studies had <200 patients available for analysis. Only one, testing 44 patients, examined a possible role between HLA-C and the risk of leukemia; no correlation was found. This issue was addressed using the large database of the IBMTR where HLA phenotypes are known for all patients.

Frequencies of 35 HLA A, B, C, and DR antigens were determined in 1834 leukemic Caucasoids to evaluate possible associations between HLA and leukemia.\(^5\) In comparison with the frequencies of HLA antigens in published controls, the frequency of Cw3 was significantly higher in 727 patients with ALL (relative risk 2.64, p < 0.0002), 665 patients with acute myelogenous leukemia (relative risk 1.92, p < 0.0007), and 442 patients with CML (relative risk 2.07, p < 0.002; p values adjusted for multiple comparisons). The frequency of Cw4 was elevated in patients with ALL (relative risk 2.01, p < 0.0003), AML (relative risk 2.06, p < 0.0002) and CML (relative risk 2.14, p < 0.0008). The frequency of Aw19 was significantly decreased in patients with AML (relative risk 0.68, p < 0.01) and CML (relative risk 0.59, p < 0.005). The overall associations between HLA and ALL, AML and CML, combined, are presented in Table V. None of the other 32 HLA antigens investigated had a statistically significant association with leukemia.

The data suggest that Cw3 and Cw4 antigens or the genes encoding them are, or are closely linked with, possible leukemia susceptibility genes. The observed increased risk of leukemia in siblings of leukemia patients may be due in part to an increased frequency Cw3 and Cw4 within the family. The findings in this study indicate that HLA C genes have hitherto unrecognized properties. Further studies are required to determine whether HLA C also will be found to be associated with other malignant and non-malignant diseases.

**Studies in progress**

Several analyses presently are in various stages of completion. The results will be submitted for publication within the 12 months. The investigators with primary responsibility for these studies and the tentative titles are listed below.

1. Robert C. Ash, USA: Bone marrow transplantation using donors other than HLA-identical siblings.
2. Kerry Atkinson, Australia: Risk factors for chronic GVHD.
5. Mortimer M. Bortin, USA: Temporal relationship between major complications of allogeneic bone marrow transplantation.
6. Robert Peter Gale, USA: Bone marrow transplantation or chemotherapy for acute myelogenous leukemia in first remission.
7. Eliane Gluckman, France: Effect of treatment regimen upon survival after bone marrow transplantation for severe aplastic anemia.
10. Alberto M. Marmont, Italy: Results of bone marrow transplantation using T cell-depleted grafts in leukemia.

Comment

The use of bone marrow transplants continues to increase rapidly. There is no generally accepted bone marrow transplant protocol; new treatment strategies are frequently introduced. The large database of the IBMTR makes analyses possible evaluating the strengths and weaknesses of these new approaches in comparable patients. This is particularly helpful in those instances when prospective clinical trials at single centers are difficult or not possible, e.g. in adult ALL. Risk factors associated with the complications of bone marrow transplantation have been identified that can be useful in estimating the risk to a particular patient, in modifying treatment and in designing appropriately stratified clinical trials. Finally, `state of the art' analyses such as presented here, provide a benchmark against which individual teams can compare their results. Teams wishing to participate in the IBMTR research program should send their request to the Statistical Center.

Acknowledgments

This 55th report from the IBMTR was prepared for the Advisory Committee: Robert Peter Gale, University of California at Los Angeles (Chairman); Kerry Atkinson, St Vincent's Hospital, Sydney; Fritz H. Bach, University of Minnesota, Minneapolis; A. John Barrett, Hammersmith Hospital, London; Dirk W. van Bekkum, Radiobiological Institute, Rijswijk; James C. Biggs, St Vincent's Hospital, Sydney; Karl G. Blume, City of Hope National Medical Center, Duarte; Mortimer M. Bortin, Medical College of Wisconsin, Milwaukee; Karel A. Dicke, M. D. Anderson Hospital and Tumor Institute, Houston; Costa Gahrton, Karolinska Institutet, Stockholm; Eliane Gluckman, Hôpital Saint-Louis, Paris; John M. Goldman, Royal Postgraduate Medical School, London; Robert A. Good, All Children's Hospital, St Petersburg; Werner Heilig, Karl Marx University, Leipzig; Roger H. Horzig, Cleveland Clinic, Cleveland; Richard Hong, University of Wisconsin, Madison; John H. Kersey, University of Minnesota, Minneapolis; Hans-Jochem Kolb, Universität München, Munich; Alberto M. Marmont, Ospedale San Martino, Genoa; Tohru Masoka, Center for Adult Diseases, Osaka; Hans A. Messner, Ontario Cancer Institute, Toronto; Richard J. O'Reilly, Memorial Sloan-Kettering Cancer Center, New York; Ray L. Powles, Royal Marsden Hospital, London; Alfred A. Rimm, Medical College of Wisconsin, Milwaukee; Olle Ringdén, Huddinge University Hospital, Huddinge; Jon J. van Rood, University Hospital, Leiden; Ciril Rozman, Barcelona University, Barcelona; Bruno Speck, Kantonsklinik, Basel; Roy S. Weiner, University of Florida, Gainesville; and Ferdinand E. Zwaan, University Medical Center, Leiden.

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References


Appendix

List of centers currently participating in the IBMTR research program

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CHAPTER 10

Current Status of Bone Marrow Transplantation

MORTIMER M. BORTIN AND MARY M. HOROWITZ

INTERNATIONAL BONE MARROW TRANSPLANT REGISTRY
Medical College of Wisconsin
Milwaukee, Wisconsin

GROWTH IN THE FIELD OF BONE MARROW TRANSPLANTATION

The International Bone Marrow Transplant Registry (IBMTR) is a voluntary working group conducting research to determine factors affecting success and failure of bone marrow transplantation. In addition to these clinical epidemiologic investigations, the IBMTR conducts occasional surveys to assess worldwide utilization of allogeneic and syngeneic bone marrow transplantation. Our previous survey covered the period 1955-1984 (1). We now report continued growth during 1985-1987 (2).

Data were reported to the IBMTR by 258 institutions in 41 countries regarding their patients who received bone marrow transplants during the period 1985-1987. To the best of our knowledge, the data represent essentially all bone marrow transplants (exclusive of autologous bone marrow transplants) performed in this 3-year interval. Between 1985 and 1987, a total of 10,887 patients received bone marrow transplants: 73% were for leukemia, 11% for other malignant diseases, 9% for severe aplastic anemia and related disorders, 3% for immune deficiency diseases, 2% for thalassemia major, and 2% for genetic, metabolic, and other rare diseases. Forty-six percent of the world's bone marrow transplants were performed in North America, 42% in Western Europe, 5% in Asia/India, 3% in Australia/New Zealand, 2% in the Midd-

![Graph](https://via.placeholder.com/150)

**Figure 1.** Linear extrapolation estimating annual number of bone marrow transplants that will be performed through 1995 (from Ref. 2).

Figure 2. Probability of leukemia-free survival for patients with acute lymphoblastic leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 3. Probability of relapse for patients with acute lymphoblastic leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 4. Principal causes of death for patients transplanted in first remission of acute lymphoblastic leukemia.
estimate predicts that approximately 7,600 patients will receive allogeneic or syngeneic bone marrow transplants in 1995.

RESULTS OF BONE MARROW TRANSPLANTATION AND FACTORS AFFECTING OUTCOME

Acute Lymphoblastic Leukemia

Comprehensive data from 1,657 patients with acute lymphoblastic leukemia treated with high-dose chemoradiotherapy and bone marrow transplants from HLA-identical sibling donors between January 1, 1980 and December 31, 1988 were reported to the IBMTR by 141 transplant centers, worldwide. The data were analyzed to determine factors associated with the probability of leukemia-free survival and the probability of leukemia relapse. The 5-year actuarial probability (±95% confidence interval) of leukemia-free survival was 44±6% for 514 patients transplanted in first complete remission (CR), 30±4% in 762 patients transplanted in 2nd-8th CR, and 14±5% in 381 patients transplanted when their disease was more advanced (Fig. 2). The 5-year actuarial probability of relapse for this same patient population was 28±6%, 50±6%, and 68±7%, respectively (Fig. 3). The principal causes of death among the 230 acute lymphoblastic leukemia patients transplanted in first remission are shown in Figure 4.

Disease status at the time of transplantation was the most important predictor of outcome in acute lymphoblastic leukemia. It should be noted that these relapse and survival curves, as well as others in this chapter, are based on raw data; they are not adjusted for other factors that might influence outcome such as patient age; preparative antileukemic, immunosuppressive conditioning regimen; method used to prevent graft-versus-host disease (GvHD); or other factors reported to be associated with outcome. It should be noted further that most of the patients transplanted in first remission were adults whereas most of the patients transplanted in second or subsequent remission were children.

In first remission adults, non-T cell phenotype, male-to-female donor-recipient sex-match, and GvHD were associated with decreased leukemia-free survival; inclusion of corticosteroids in the regimen to prevent GvHD was associated with a higher probability of leukemia-free survival (3). Variables associated with decreased leukemia-free survival among second remission transplants were age ≥16 and relapse occurring while on therapy (3). Variables associated with increased probability of relapse were similar for first and second remission transplants and included GvHD prophylaxis without methotrexate (4), and the absence of GvHD (3). In first remission transplants, leukocyte count ≥50 x 10⁹/l at diagnosis was also associated with an increased risk of relapse; in second remission transplants, relapse while receiving chemotherapy was also associated with increased posttransplant relapse. These data emphasize the importance of both disease and transplant-related variables in predicting outcome after bone marrow transplantation in acute lymphoblastic leukemia. The data may be useful in helping to explain differences among studies, to design prospective controlled clinical trials, and to identify persons most likely to benefit from bone marrow transplantation.

Acute Myelogenous Leukemia

Comprehensive data regarding 2,018 patients transplanted for acute myelogenous leukemia were reported to the IBMTR by more than 151 transplant centers. All were treated with high-dose chemotherapy with or without total body radiation. All received bone marrow transplants from their HLA-identical siblings during the period January 1, 1980 to December 31, 1988. Disease status at the time of transplant was the most important factor influencing the probability of leukemia-free survival (Fig. 5) and relapse (Fig. 6). The 5-year actuarial probability of leukemia-free survival was 50±3% for 1,259 patients transplanted in first CR, 27±6% in 301 patients transplanted in 2nd-4th CR, and 18±5% in 458 patients with more advanced disease. The 5-year probability of relapse in this same patient population was 21±3%, 45±10%, and 63±7%, respectively (Fig. 6). The principal causes of death among the 526 patients transplanted in first remission are shown in Figure 7.

A controversial issue in the management of patients with acute myelogenous leukemia who have achieved first remission and who have an HLA-identical sibling donor is whether to continue chemotherapy or to perform a bone marrow transplant. The reported 5-year probability of leukemia-free survival with chemotherapy ranges from 20-50%; in most reports, HLA-identical sibling bone marrow transplants are associated with 5-year leukemia-free survival rates of 35-60%. This problem of overlapping results is further complicated by censoring patients who relapse before transplants can be done, and by selective data reporting. Some centers recom-
Figure 5. Probability of leukemia-free survival for patients with acute myelogenous leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 6. Probability of relapse for patients with acute myelogenous leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 7. Principal causes of death for patients transplanted in first remission of acute myelogenous leukemia.
mend transplants only for patients for whom a poor response to chemotherapy is predicted.

To evaluate the wisdom of this latter strategy, data from 704 recipients of HLA-identical sibling transplants for acute myelogenous leukemia in first remission were analyzed (5). Improved leukemia-free survival was associated with younger age and lower white blood cell counts at diagnosis. These prognostic variables are similar to those reported to affect outcome after chemotherapy. These findings suggest that a strategy of treatment assignment based on risk factor analysis is unlikely to resolve the controversy of transplant versus chemotherapy for AML in first remission.

**Chronic Myelogenous Leukemia**

Data were analyzed for 1,613 patients with chronic myelogenous leukemia who were treated with high-dose chemotherapy and/or total body irradiation followed by bone marrow transplantation from HLA-identical sibling donors. The transplants were performed between January 1, 1980 and December 31, 1988 by 143 teams, worldwide. As in acute leukemia, disease status was an important predictor of outcome. The 5-year probability of leukemia-free survival was 42±5% for 980 patients transplanted in first chronic phase; 21±8% for 445 transplanted in accelerated phase; and 15±6% for 188 transplanted in blast phase (Fig. 8). The 5-year probability of relapse was 25±6%, 48±11%, and 52±13%, respectively (Fig. 9). The principal causes of death after transplantation in chronic phase are shown in Figure 10.

Earlier multivariate analyses of 405 patients transplanted in first chronic phase disclosed that probability of relapse was higher for recipients of T cell depleted transplants in comparison with recipients of non-T cell depleted bone marrow (relative risk 5.4, p<0.0001) (6). The probability of relapse was also higher for patients who did not develop chronic GvHD in comparison with patients who did (relative risk 3.1, p<0.01) (6). Despite these problems, bone marrow transplantation in chronic phase, in contrast to any other therapy, offers some patients the possibility of prolonged leukemia-free survival (Fig. 8). Once blast transformation has occurred, the results of transplantation are significantly poorer. Therefore, the opportunity for cure should not be lost by prolonged delay and the attendant risk of transformation, especially in younger patients with an HLA-identical sibling.

**Severe Aplastic Anemia**

Comprehensive data from 971 patients with severe aplastic anemia treated with high-dose chemotherapy with or without radiotherapy and bone marrow transplantation from HLA-identical donors between January 1, 1980 and December 31, 1988 were reported to the IBMTR by 124 transplant teams. The 5-year actuarial probability of survival was 59±3%. Both age (Fig. 11) and acute GvHD (Fig. 12) had highly significant associations with survival following bone marrow transplants from HLA-identical sibling donors. The 486 patients aged 19 or under had a 5-year probability of survival of 67±5%; the probability was 51±5% among the 485 older patients (p<0.0001). The 5-year probability of survival among 559 patients who developed no or only mild acute GvHD was 80±4%; this was significantly higher (p<0.0001) than the 39±6% probability of survival among 334 patients who developed moderate to severe acute GvHD. The principal causes of death among the 364 severe aplastic anemia patients who died after transplant are shown in Figure 13.

A major cause of treatment failure following bone marrow transplantation for severe aplastic anemia is no or only transient engraftment of the donor bone marrow. Graft failure was analyzed in 625 patients receiving allogeneic bone marrow transplants from HLA-identical sibling donors (7). Sixty-eight (11%) had graft failure. Second bone marrow transplants were successful in achieving extended survival in 16 of 27 patients with transient initial engraftment but in none of 10 patients with no sign of engraftment after the first transplant. The major factors associated with reduced risk of graft failure were use of radiation for pretransplant immune suppression and use of cyclosporine (CsA) rather than T cell depletion of the donor marrow or methotrexate for prophylaxis against GvHD. Among 266 patients prepared for transplantation with cyclophosphamide alone, the risk of graft failure was increased in patients who had received transfusions previously and reduced in those who received corticosteroids for previous therapy. Neither cell dose nor administration of donor buffy coat cells affected the probability of engraftment. Although use of radiation for conditioning reduced graft failure, survival was not improved. Posttransplant treatment with CsA and avoidance of pretransplant blood transfusions were associated with improved survival.
Figure 8. Probability of leukemia-free survival for patients with chronic myelogenous leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 9. Probability of relapse for patients with chronic myelogenous leukemia following high-dose chemotherapy with or without total body radiation and bone marrow transplantation from HLA-identical siblings based on disease status at time of transplant.

Figure 10. Principal causes of death for patients transplanted in first remission of chronic myelogenous leukemia.
Figure 11. Probability of survival for patients with severe aplastic anemia following bone marrow transplantation from HLA-identical sibling donors based on age of patient in years.

Figure 12. Probability of survival for patients with severe aplastic anemia following bone marrow transplantation from HLA-identical sibling donors based on severity of acute GvHD posttransplant.

Figure 13. Principal causes of death for patients transplanted in first remission of severe aplastic anemia.
These data point to several modes of treatment associated with reduced graft failure. In conjunction with information regarding the impact of these therapies on other transplant-related complications, this suggests that some measures may improve the outcome of bone marrow transplantation for severe aplastic anemia.

Regimens containing CsA appear to have an advantage over methotrexate. If possible, transfusions should be withheld or minimized prior to the initiation of the immune suppressive preparative regimen, especially if regimens without radiation are to be used.

**Summary**

Utilization of bone marrow transplantation as a therapeutic modality continues to increase. More and more institutions are initiating bone marrow transplant programs. During the 33-year period between 1955 and 1987, more than 20,000 patients received allogeneic bone marrow transplants; more than 50% of these were performed in the 3 years, 1985-1987. Transplantation is an effective therapy for acute leukemia; in some instances it is the preferred treatment. In chronic myelogenous leukemia, severe aplastic anemia, and some genetic and immune deficiency diseases, bone marrow transplantation provides the only possibility for cure. Bone marrow transplantation is associated with serious problems such as graft-versus-host disease (GvHD), graft failure, interstitial pneumonitis and, until recently, the requirement for an HLA-identical sibling donor. In the past few years, an increasing number of transplants have been performed using HLA-partially matched related or unrelated donors with some success, the level of which is yet to be determined. The development of acute GvHD (8) and interstitial pneumonitis (9,10) can often be predicted by risk factor assessment. Special precautions can then be taken for patients at high risk of these complications. In this report, current data from the International Bone Marrow Transplant Registry were summarized and several risk factors affecting outcome were identified.

**References**

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C. AUSTRALIA/ASIA

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| Royal Alexandra Hospital                 | Camperdown| Australia | Kanazawa University          | Kanazawa | Japan |
| St. Vincent’s Hospital                   | Darlinghurst| Australia | Kyoto University             | Kyoto    | Japan |
| Royal Children’s Hospital                | Parkville | Australia | Nish University              | Mie      | Japan |
| Royal Melbourne Hospital                 | Parkville | Australia | Shimizu University          | Nagano-kan| Japan |
| Royal Perth Hospital                     | Perth     | Australia | National Nagoya Hospital     | Nagoya   | Japan |
| Alfred Hospital                          | Perth     | Australia | Nagoya First Red Cross Hospital | Nagoya | Japan |
| Royal Brisbane Hospital                  | Queensland| Australia | Nagoya University            | Nagoya   | Japan |
| Prince of Wales Children’s Hospital      | Randwick | Australia | Branch Hospital Nagoya University | Nagoya | Japan |
| Westmead Centre                          | Westmead | Australia | Nagoya Second Red Cross Hospital | Nagoya | Japan |
| Queen Elizabeth Hospital                 | Woodville | Australia | Niigata University           | Niigata   | Japan |
| Beijing Medical University               | Beijing   | China   | Niigata University/Medicine I  | Niigata   | Japan |
| Lanzhou General Hospital                 | Lanzhou   | China   | Center for Adult Diseases     | Osaka     | Japan |
| Tata Memorial Hospital                   | Bombay    | India   | Osaka University              | Osaka     | Japan |
| Akita University                         | Akita     | Japan   | Kinki University/Third Department | Toba    | Japan |
| University of Chiba                      | Chiba-city| Japan   | Matsudo City Hospital         | Tochigi    | Japan |
| Kokura Memorial Hospital                 | Fukuoka   | Japan   | Jichi Medical School          | Tokyo     | Japan |
| National Kyusyu Cancer Center            | Fukuoka   | Japan   | Niho University               | Tokyo     | Japan |
| Hiroshima Red Cross Hospital             | Hiroshima | Japan | Tokyo University              | Tokyo     | Japan |
| Hiroshima University                     | Hiroshima | Japan | Tokyo Medical and Dental University | Tokyo | Japan |
| Hyogo College of Medicine                | Hyogo     | Japan   | Tokyo Women’s College         | Tokyo     | Japan |
| Central Hospital of Kobe City            | Hyogo     | Japan   | University of Tokyo           | Tokyo     | Japan |
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Management of aplastic anaemia — P. Jacobs, N. Novitzky

P. Jacobs, Professor and Head, N. Novitzky, Specialist Haematologist, University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Observatory

The clinical syndrome of bone marrow failure is characterised by anaemia, neutropenia and thrombocytopenia. Classically, damage to haematopoietic stem cells by a variety of mechanisms results in pancytopenia and a variable degree of haematopoietic hypocellularity, best assessed by examination of the bone marrow trephine biopsy specimen. Internationally accepted criteria exist to define the most severe form of this lesion, known as aplastic anaemia, and its distinction from lesser degrees of haematopoietic failure is of practical importance, since therapeutic options differ depending on whether hypoplasia or aplasia is present.

Diagnostically, the cardinal clinical rules apply. A meticulous history must be taken with special attention to other involved family members, prior viral diseases or exposure to radiation and a wide range of drugs, including cytotoxic and anti-inflammatory agents, certain antibiotics, and potentially myelotoxic industrial toxins such as benzene. While the physical examination should be carefully conducted, these patients are, with the exception of those with congenital lesions, usually unremarkable apart from some degree of pallor, thrombocytopenic purpura or the occurrence of pyrexia associated with infection.

The key investigations are haematological. The full blood count will define the extent of the pancytopenia and, while bone marrow aspiration is of limited value, the proper interpretation of an adequate trephine biopsy specimen is usually diagnostic. Inflammatory changes have been well described and stated to have both aetiological and prognostic significance. The most striking morphological feature is a virtual absence of recognisable haematopoietic tissue, although the stromal elements including adipocytes, plasma cells and lymphocytes may be prominent.

The pathogenesis is variable and in certain cases immune mechanisms may be operative while in others the micro-environment may be functionally abnormal. Not surprisingly, the range and complexity of diagnostic techniques has widened to include immunological and culture studies in order to select subgroups of patients for different forms of treatment.

The therapeutic options are influenced by prior supportive therapy, age, and the availability of a donor for bone marrow allografting. It is unfortunately true that many patients are referred only after trials of haematinics and transfusion of red cells or other blood components. While such management may be well intentioned, it can immunise the recipient and may even compromise the outcome of therapy. It is therefore emphasised that immediate referral to a specialised centre is desirable.

Once a reliable diagnosis of aplastic anaemia has been established, what might constitute a reasonable approach to management? In those patients where drug-related lesions, usually immunological, are responsible for the aplasia, these will generally resolve spontaneously within 2–3 weeks and are excluded from further consideration.

Bone marrow transplantation remains the preferred form of treatment for patients under the age of 40 years who have an HLA identical and MLC non-reactive sibling and patients who have not received blood transfusion are regarded as having an excellent probability of long-term survival and a normal life. This approach needs to be tempered with the recognition that successful transplantation may be complicated by acute or chronic graft-versus-host disease (GVHD), particularly in patients with aplastic anaemia. To try to overcome this difficulty, ex vivo T-lymphocyte depletion has been employed and it now has to be established to what extent this modification may increase the risk of non-engraftment or early rejection. It may be speculated that T-lymphocyte depletion removes a specific suppressor population from the donor marrow so that partial autologous reconstitution may occur following infusion, which leads to the emergence of a cytotoxic clone with the capacity to recognise and reject the new graft. It is a matter of pressing importance that this hypothesis be critically tested to determine whether intensified conditioning regimens or post-transplantation immunosuppression is needed to influence engraftment favourably and to contribute to further reduction of GVHD.

An alternative therapeutic option is suggested by the observation that in some transplanted patients clinical improvement occurs despite allograft rejection, and this can be correlated with autologous reconstitution. This phenomenon raises the possibility that intensive immunosuppression with high-dose corticosteroids, antilymphocyte globulin, antithymocyte globulin, or monoclonal antihuman T-cell antibodies may offer alternative therapeutic regimens. Although the mechanism of action of these products is not clearly defined, response rates appear to be in
excess of 50%, being highest when treatment commences as soon as possible after diagnosis and in patients where adequate doses are given. It should be noted that this group of products is difficult to standardise and there is at least some evidence suggesting that apparently similar biological products may vary in their activity.

In these circumstances it would seem reasonable to offer the young and previously non-transfused patient (with a suitable donor) bone marrow allogenic grafting following T-lymphocyte depletion as the first option. However, such patients would be in the minority, particularly since compatible siblings are limited and most individual clients reach referral centres after varying degrees of immunosuppression consequent upon red cell or blood component infusion. In these patients a combination of high-dose corticosteroids combined with an adequate dose of biologically active antilymphocyte globulin would seem appropriate. It does, however, need to be borne in mind that allogeneic platelet support is unavoidable during this period of treatment because of further falls in platelet count, and that there may be additional discomfort of immune-complex arthralgia. It is our experience that an initial clinical and haematological response can be detected as early as 4 weeks, although the median time to response is reached in 12 weeks. Non-responding patients might then be considered for allogeneic bone marrow transplantation if this option has not already been exercised or, as a second choice if no donor is available, a second course of antilymphocyte globulin could be given with the likelihood that a significant response rate might still be achieved. Non-responding patients should be offered splenectomy, since this can be carried out with acceptable risk, may modify the course of the disease, and can in any case be expected to reduce dependence upon blood transfusion.

REFERENCES


Opsomming

Die hantering van aplastiese anemie

Beenuurversing kom, soos hartversing of nierversing, in wisselende grade voor. Die ernstigste graad is bekend as aplastiese anemie en dit is belangrik dat hierdie vorm van minder ernstige grade van versing ondersteek word omdat die therapie verskil.

Hierdie artikels beskryf die diagnose, wat op 'n nouteletende geskiedenis en hematologiese onderzoek gegrond moet word. Beenuurasperasie se waarde is beperk en 'n toereikende treinbeeldspreke is gewoonlik diagnosties. Die opvolgende stemeken van die biopsiemuster is 'n feitlike afwasging van herkenbare hematopiese weefsel. Hierbenevans kan daar d.m.v. immunologie en kultuurstudie moontlike subgroep paie se onderzoek word vir verskillende behandelaans. 

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SA Joernal van Voortgesette Mediese Onderzoek 5 Januari 1987
SPECIAL REPORT

The EBMT activity survey 2006 on hematopoietic stem cell transplantation: focus on the use of cord blood products

A Gratwohl1, H Baldomero1, K Frauendorfer2, V Rocha3, J Apperley4 and D Niederwieser5, for the Joint Accreditation Committee of the International Society for Cellular Therapy ISCT and the European Group for Blood and Marrow Transplantation EBMT (JACIE)

1Hematology, Department of Medicine, University Hospital Basel, Basel, Switzerland; 2Institute for Operations Research and Computational Finance, University of St Gallen, St Gallen, Switzerland; 3Eurorcord, Department of Haematology, Hopital St Louis, Paris, France; 4Department of Haematology, Hammersmith Hospital, London, UK and 5Division of Hematology/Oncology, Department of Internal Medicine, University Hospital, Leipzig, Germany

This report describes the hematopoietic stem cell transplantation (HSCT) activity in Europe in 2006 by indication, donor type and stem cell source. It illustrates differences compared to previous years and concentrates on the use of cord blood transplants. In 2006, there were 25,050 first HSCT, 9,661 allogeneic (39%), 15,389 autologous (61%) and 3,690 additional re- or multiple transplants reported from 605 centers in 43 participating countries. Main indications were leukemias (7,963 (32%); 85% allogeneic)), lymphomas (11,469 (56%); 89% autologous)), solid tumors (1,564 (6%); 95% autologous)), non-malignant disorders (1,242 (5%); 90% allogeneic)) and non-classified ‘others’ (112 (1%)). There was an increase in allogeneic HSCT of 9% when compared to 2005, while autologous HSCT numbers remained similar. There were 544 allogeneic cord blood HSCT, which corresponds to 5% of all allogeneic HSCT. The majority, 67%, were used for patients with leukemia. The highest percentage of cord blood transplants, 27%, was seen for inherited disorders of metabolism. No autologous cord blood transplants were reported. The highest increase in allogeneic HSCT was observed for AML, which comprises 31% of all allogeneic HSCT. Numbers of autologous HSCT remained similar in most main indications. This data provide an update of the current HSCT experience in Europe.

Bone Marrow Transplantation (2008) 41, 687–705; doi:10.1038/sj.bmt.1705956; published online 17 December 2007

Keywords: hematopoietic stem cell transplantation; Europe; transplant rates; cord blood transplants; unrelated transplants

Introduction

In 1990, the European Group for Blood and Marrow Transplantation (EBMT) introduced the activity survey as a novel instrument to capture comprehensive information on transplant numbers and to distribute this information rapidly. All EBMT members and affiliated teams report since then on an annual basis their number of patients transplanted by indication, stem cell source and donor type. By now, it has evolved as a mandatory self-reporting system and forms an integral part of the comprehensive quality assurance program Joint Accreditation Committee of the ISCT and EBMT (JACIE) (http://www.JACIE.org). It provides the basis for counseling on the individual patient level as well as for health-care institutions and administrative agencies in the field of stem cell transplantation.

Besides a report of the data from the past years, the activity survey has focused each year on a different aspect of hematopoietic stem cell transplantation (HSCT). This includes the description of trends, the introduction of new techniques or the changes in techniques as well as the analysis of economic factors related to HSCT. In 2006, the focus was on the use of cord blood as stem cell source. The first report of a successful cord blood transplant for a patient with Fanconi's anemia gave the basis for the building of cord blood banks all over the world. By 2007, nearly 300,000 cord blood products are available for use together with the more than 11 million unrelated donors from the 75 stem cell donor registries worldwide (www.bmdw.org). Carefully conducted retrospective analyses have confirmed the value of cord blood transplants under well-defined conditions compared to other family donor transplants or unrelated transplants. The present report gives now most recent information on the use of cord blood transplants in Europe in 2006 within the context and in comparison with other stem cell sources.

Patients and methods

Data collection and validation

Participating teams reported their data for 2006 by indication, stem cell source and donor type as listed in
Table 1. Data were validated by three independent systems: through confirmation by the reporting team, which received a computer printout of the entered data; by selective comparison with MED-A data sets in the ProMISE data capture system of the EBMT (www.mbsf.nl/Promise) and by cross-checking with national registries where they exist. Onsite visits of selected teams were part of the quality control program (www.jacie.org).

Teams
In total, 615 active transplanting teams in 43 countries (38 European and 5 affiliated countries) were contacted for the 2006 report, of which 605 reported their numbers. This corresponds to a 98% return rate of active teams and includes 498 active EBMT member teams reporting to the survey. Ten teams known by the investigators to have been performing HSCT in 2006 were also contacted, but chose not to reply or, for unknown reasons, failed to reply in spite of several efforts to reach them. No major transplant team in Europe is missing from this list. All contacted teams are listed in the Appendix in alphabetical order according to country, city and EBMT center code. We received information that in 2006 no blood or marrow transplants were performed in the following European countries: Albania, Andorra, Armenia, Georgia, Liechtenstein, Malta, Moldova, Monaco, San Marino, and The Vatican. Non-European countries include, by EBMT tradition, Algeria, Iran, Israel, Saudi Arabia, South Africa and Tunisia. Their data are in part included in some of the analyses.

Definitions
Transplant numbers. The EBMT survey focused, as in previous years, on the number of patients treated for the first time with HSCT. Information on additional transplants, for instance, a second, third or fourth HSCT in a patient with a previous HSCT was collected by disease category only for those patients with a planned double autologous after autologous transplants; for all other situations this information was collected generically only. The following definitions were used:14 Re-transplants (autologous or allogeneic) were defined as an unplanned HSCT for rejection or relapse after a first HSCT. Multiple transplants were defined as being part of a planned double or triple autologous or allogeneic transplant protocol. Information on stem cell source was collected as bone marrow, peripheral blood or cord blood. Combined bone marrow, peripheral blood and cord blood transplants were reported as peripheral blood HSCT. The possibility for reporting cord blood transplants by indication and donor type was introduced for the first time in this year's survey. Information on reduced intensity conditioning was collected as a total for each team only and not for individual transplants. Definitions for reduced intensity conditioning (RIC) HSCT followed the recently published definitions.

Transplant rates. Transplant rates were computed as number of HSCT per 10 million inhabitants as previously defined.2 Transplant rates refer to the number of transplants in a given country compared to its own population. The survey cannot make adjustments for patients who cross borders and receive their HSCT in a foreign country. Population data were obtained from the US census office (http://www.census.gov).

Economic factors. Economic factors considered in the analysis followed the previously defined rules. Countries were categorized by their gross national income per capita according to the World Bank definitions into high-income (Australia, Belgium, Cyprus, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Netherlands, Norway, Portugal, Slovenia, Spain, Sweden, Switzerland and UK), middle-income (Croatia, Czech Republic, Estonia, Hungary, Latvia, Lithuania, Poland and Slovakia) and low-income countries (Azerbaijan, Belarus, Bosnia and Herzegovina, Bulgaria, Macedonia, Romania, Russia, Serbia and Montenegro, Ukraine and Turkey). The latter category refers to the World Bank definition of 'lower middle income' (http://www.worldbank.org) as previously used.

Non-European countries that traditionally participate in the EBMT activity survey (Algeria, Iran, Israel, Saudi Arabia, South Africa and Tunisia) are included in the overall data presentation. They were not included in the analysis on economic factors. The same applies to Iceland and Luxemburg, because of some missing data over the time span.

Results
Participating teams
Of the 605 teams reporting HSCT in 2006, 361 (60%) did both allogeneic and autologous transplants; 227 (37%) restricted their activity to autologous, 8 teams (1%) to allogeneic transplants only. Nine teams (2%) reported having performed no transplants in 2006.

In total, 211 teams (34%) did fewer than 20 HSCT in 2006, 210 teams (35%) between 20 and 50 HSCT, 132 teams (22%) between 50 and 100 HSCT and 52 teams (9%) >100 HSCT.

In total, 136 teams reported at least one cord blood HSCT in 2006 and 23 teams reported >5.

Number of HSCT in 2006
First transplants 2006. A total of 25050 first transplants, 9661 (39%) allogeneic and 15389 (61%) autologous were carried out in 2006 (Table 1). Overall, this corresponds to a slight increase in the number of HSCT compared to 2005, when there were 24168 first transplants. Number of allogeneic HSCT increased by 9% from 8890 in 2005 to 9661 in 2006, while the number of autologous HSCT remained similar; 15278 in 2005 and 15389 in 2006.

Additional transplants 2006
There were 1557 re-transplants (772 allogeneic/785 autologous) and 2133 additional planned multiple transplants (711 allogeneic/2062 autologous). Thus, there were a total of 28740 HSCT procedures; 10504 allogeneic (37%) and 18236 autologous (63%) transplants were performed in 2006. This corresponds to an overall increase of 93 re-transplants (74 allogeneic and 19 autologous) or 6% as

Bone Marrow Transplantation
Table 1  Number of patients treated in Europe during the year 2006 with a first hematopoietic stem cell transplant listed by indication, donor type and stem cell source

<table>
<thead>
<tr>
<th>Family</th>
<th>Donor source</th>
<th>Non-id</th>
<th>Twin</th>
<th>Unrelated</th>
<th>BM</th>
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<td>BM PBPC</td>
<td>Cord</td>
<td>BM PBPC</td>
<td>Cord</td>
<td>BM PBPC</td>
<td>Cord</td>
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<td>18</td>
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<td>118</td>
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<td>18</td>
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<td>31</td>
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<td>99</td>
<td>436</td>
<td>4</td>
<td>11</td>
<td>32</td>
<td>952</td>
</tr>
</tbody>
</table>

Abbreviations: BM = bone marrow; MDS = myelodysplastic syndromes; MM = multiple myeloma; MPS = myeloproliferative disorders; PBPC = peripheral blood progenitor cells; SAA = severe aplastic anemia; sec. AL = secondary acute leukemia; thal = thalassemia.

«These differences relate to all transplants (Figure 1a), to allogeneic HSCT (Figure 1b) and to autologous HSCT (Figure 1c). Differences between Eastern and Western European countries have been previously reported. Of interest to note is that countries with similar total transplant rates had similar transplant rates for allogeneic HSCT as well as for autologous HSCT.»

Transplant rates in 2006. There were marked differences in transplant rates between European countries and countries affiliated with EBMT as presented in Figure 1.

These differences relate to all transplants (Figure 1a), to allogeneic HSCT (Figure 1b) and to autologous HSCT (Figure 1c). Differences between Eastern and Western European countries have been previously reported. Of interest to note is that countries with similar total transplant rates had similar transplant rates for allogeneic HSCT as well as for autologous HSCT.

Disease indications

Indications for HSCT in 2006 are listed in detail in Table 1. Main indications were lymphoproliferative disorders with 14,169 patients (56%), 1,597 patients with allogeneic HSCT.

Compared to 2005. Regarding the planned double autologous-allogeneic HSCT, there were a total of 531 procedures. Compared to 2005, when there was a total of 671 planned double autologous-allogeneic HSCT, this corresponds to a decrease of 21%. Main indications for the planned double transplant programs were, as in the previous year, multiple myeloma, non-Hodgkin’s lymphoma and Hodgkin’s disease.17,20

Bone Marrow Transplantation
Figure 1 Transplant rates (number of HSCT per 10 million inhabitants) in European countries in 2006. (a) All HSCT combined, (b) allogeneic HSCT only and (c) autologous HSCT only.

(11%), 12,572 with autologous HSCT (89%); leukemias with 7,963 patients (32%), 6,784 patients with allogeneic (85%), 1,179 with autologous (15%) HSCT; solid tumors with 1,564 patients (6%), 85 with allogeneic HSCT (6%), 1,479 with autologous HSCT (95%) and non-malignant disorders with 1,242 patients (5%), 1,115 with allogeneic HSCT (90%), 127 with autologous HSCT (10%). The latter, autologous HSCT for non-malignant disorders predominantly include patients (119) with autoimmune disorders. An additional 112 patients (1%), 80 with allogeneic HSCT and 32 with autologous HSCT were listed as 'other indications'.

Stem cell source
Of the 15,389 autologous first transplants, 256 (2%) were bone marrow derived, 15,133 (98%) from peripheral blood stem cells or from combined bone marrow and peripheral blood stem cell transplants (Table 1). Of the 9,661 allogeneic first transplants, 24% were bone marrow, 71% were peripheral blood and 5% were cord blood transplants (Figure 2). This corresponds to a slight decrease in the proportion of peripheral blood as stem cell source compared to the 74% in 2005. The proportion of peripheral blood as stem cell source varied depending on donor type. It was 72% for HLA-identical sibling donor transplants, 67% for unrelated donors, 81% for HSCT from other family members and, 74% for twin donors. Within allogeneic HSCT, the only disease indications with more bone marrow than peripheral blood donors were stem cell source were bone marrow failure syndromes (57% bone marrow) and congenital disorders (58% bone marrow) (Figure 2a). The proportion of main indications varied as well within the three stem cell sources. Non-malignant disease represented about a quarter of all indications for bone marrow and cord blood, but only a small fraction among the peripheral blood transplants (Figure 2b).

Donor type
For the 9,661 allogeneic first transplants, HLA-identical siblings were used as donors for 4,838 (50%) of the recipients, other family members for 5,39 (5.5%) of the recipients, a syngeneic twin for 43 (0.5%) of the recipients and an unrelated volunteer donor for 4,241 (44%) of the recipients.
Conditioning
Numbers of RIC HSCT continued to increase from 3301 in 2005 to 3350 in 2006 at the same rate as allogeneic HSCT. They were used in 34% of all allogeneic HSCT. This information is collected only in a generic way; no information on disease distribution is possible by the activity survey.

Cord blood transplants
A total of 544 allogeneic HSCT were cord blood transplants compared to 395 in 2005, 283 in 2004 and 86 in 1997 when this item was introduced into the activity survey. The development over the last 10 years, and the massive increase in the last four years, is illustrated in Figure 3. This increase was largely observed in countries of the high-income World Bank category. Of the 507 cord blood HSCT reported as first transplants, the majority, 458 (90.5%), were from unrelated donors. There were 45 (9%) HLA-identical and 4 (0.5%) non-identical family transplants. There were no autologous cord blood transplants reported.

Overall, the 544 cord blood transplants correspond to 5% of all allogeneic HSCT. However, there were marked differences in the use of cord blood depending on disease indication (Table 2). Of the 507 cord blood HSCT reported by indication, a higher proportion was for non-malignant disorders (total 11%), specifically for congenital bone marrow failure syndromes (7%), immune deficiencies (15%) or inherited disorders of metabolism (27%). Cord blood was rarely used for solid tumors (4%) and lymphoproliferative disorders (2%). Cord blood was used
more frequently in advanced leukemias than in early leukemia’s.

There were also major differences in the use of cord blood HSCT between the European countries. This refers to absolute numbers compared to the population (cord blood transplant rates) (Figure 4) as well as to the proportion of cord blood as stem cell source among the allogeneic HSCT (data not shown).

Donor lymphocyte infusions
There were 1920 patients reported as having received donor lymphocyte infusions in 2006. This corresponds to about two-thirds of the number of reported patients with RIC HSCT. No information on the disease indication of those patients with DLI is available from the activity survey.

Major changes over the last decade
Allogeneic HSCT. The ongoing increase in allogeneic HSCT continued at the same rate as has been observed over the last decade for almost all indications (Figure 5). Clear increases were seen in the acute leukemias, as illustrated for AML (Figure 5a) and ALL (Figure 5b). There were stable numbers for CML and lymphoproliferative disorders when compared to 2005.

Autologous HSCT. In contrast to the development in allogeneic HSCT, numbers in general remained stable for autologous HSCT overall (Figure 6) as illustrated for plasma cell disorders (PCD) (Figure 6a). However, the continuing decline in autologous HSCT for ALL is still evident (Figure 6b).

Discussion
This report presents the current state of the art of HSCT in Europe in 2006. It documents the diversity of the procedure, which includes autologous and allogeneic stem cells from the three main sources, bone marrow, peripheral blood and cord blood for a broad range of malignant and non-malignant disorders. Allogeneic HSCT continue to increase for most indications in all countries and in countries with high and middle income by World Bank categories. This increase is most pronounced in patients with acute leukemia and is observed in related and unrelated HSCT as well. In contrast to allogeneic HSCT, numbers of autologous HSCT remained similar in most disease indications when compared to 2005.

For the first time since the introduction of the EBMT activity survey, detailed information was collected concerning cord blood transplants. The data confirm the increasing use of cord blood in general. They permit a rapid presentation of current use by indication and donor type. The data show marked differences in cord blood use between European countries. These differences are not explained solely by economic factors such as gross national income per capita or World Bank category. Most likely, these differences reflect the activities of the major cord blood banks in Europe and the impact of their leaders on the transplant activities with their countries.

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non-malignant disorders, absence of GvHD is never deleterious since no graft-versus-disease effect is required.\textsuperscript{11}

The most frequent use of cord blood overall was for acute leukemias. The rapid increase in use might be due to the introduction of new technologies, for example, the use of double cord blood products to increase the number of stem cells given or the use of combined haploidentical cord blood transplants.\textsuperscript{94, 95} The survey does not provide data on these technologies; reports from the registries need to be analyzed to confirm this hypothesis. The fact that more cord blood HSCTs were used for advanced acute leukemias than for early leukemias might reflect this attitude and might reflect some of the reservations concerning general use of cord blood HSCT instead of an HLA-identical sibling or well-matched unrelated HSCT. It is also important to note that none of the teams reported an autologous cord blood HSCT in 2006.

As usual, the activity survey does not provide any outcome data. This is not the purpose of this data collection, whose focus is on the rapid dissemination of
trends. Outcome data will be reported later and elsewhere.
In contrast, this data give a clear overview on the status quo in 2006 and on the developments to be expected in the near future. As such, they provide a basis for patient counseling and health-care planning.

Acknowledgements

The cooperation of all participating teams and their staff (listed in the Appendix), the EBMT Co-ordination office; Barcelona (F. McDonald, E. McGrath, SM. Jones and EJ. Mac Huckle), Paris (V. Chernel, C. Kenzey, C. Durand and NC Gorin), London (C. Ruiz de Elvira, S. Hewerdine, S. de Souza and N. Fortin-Robertson), the Austrian Registry (H. Grinix and B. Lindner), the Czech Registry (K. Beresina and M. Tunkova), the French Registry SFGM (D. Blaise, C. Raffoux and Z. Chid), the German Registry (H. Ottinger, K. Fuchs, C. Müller, S. Allgaier and A. Müller), the Italian Registry (A. Bacigalupo, R. Oneto and B. Bruno), the Dutch Registry (A. Schuitjenberg, A. Biezen, M. Smeets and R. Brand), the Spanish Registry (J. Rifon, A. Cedillo and J. López), the Swiss Registry (U. Schanz, H. Baldomero and E. Huuffeind), the Turkish Registry (G. Gurman, M. Arat, F. Arpaci and M. Eriem) and the British Registry (C. Craddock, J. Cornish, K. Towson and M. Wilson) is greatly appreciated. We also thank S. Röckl for excellent secretarial assistance, as well as L. John for technical assistance with data management. The study was supported in part by the European Leukemia Net LSH-2002-2.2.0-3, by a grant from the Swiss National Research Foundation, 3200/BO-118176 the Swiss Cancer League, the Regional Cancer League, Foundation Cord Blood Bank Basel, and the
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References


Appendix 2006

List of transplant centers in 2006

(Total first HSCT (total all HSCT) N allogeneic first HSCT/N autologous first HSCT)

Albania: no report

Andorra: no report

Armenia: no report

Algeria: (one center): 155 (162) 100/55

Alger: Centre Pierre et Marie Curie, CIC 703, R Hamla 155 (162) 100/55
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Austria: (13 teams: 315 (291) 133/182)
Graz, Karl Franz University Hospital (onco), CIC 278 (0 (0) 0/0)
Graz, Karl Franz University Hospital (hem), CIC 308, W Linkesch
(51 (55) 25/26)
Graz, Universitäts-Kinderklinik (hem, onco), CIC 593, Ch Urban
(4 (8) 1/3)
Innsbruck, Universitätsspital (hem, onco), CIC 271, G Gastl, D
Nachbaur (54 (66) 34/20)
Klagenfurt, General Hospital Klagenfurt, D Geisler, M Heistering
(12 (12) 0/12)
List, AG Krankenhaus (hem), I Medizin, MA Fridrich (5 (5) 0/5)
List, AOK der Elisabethinen, Internal Medicine, CIC 594, D Lutz,
O Krieger (36 (47) 16/20)
Salzburg, LKA Salzburg (onco), CIC 356, R Greil (27 (40) 0/27)
Salzburg, AKH, Universitätsklinik für Innere Medizin I (onco), CIC
227, HT Greinix, P Kalb (67 (75) 39/28)
Vienna, St Anas Kinderklinik (hem, onco), CIC 528, H Gudner,
C Peter (30 (35) 18/12)
Vienna, Haushof-Krankenhaus (hem, onco), CIC 743, E Koller
(8 (12) 0/8)
Vienna, Donauklinik, CIC 767, W Horntberger (4 (5) 0/4)
Vienna, Wilhelmstiftspital (hem, onco), CIC 828, H Ludwig
(17 (31) 0/17)

Azerbaijan: (one team: no report)
Baku, Azerbaijan Central Clinical Hospital, CIC 186, S Dincer
(no report)

Belarus, Republic of: (two teams: 108 (114) 28/80)
Minsk, Belorusus Center (hem, onco, peda), CIC 591,
O Alcinkiva (42 (45) 19/23)
Minsk, Hospital no. 9, N Mikanovich (66 (69) 9/57)

Belgium: (21 teams: 584 (667) 253/331)
Antwerpen, Stuivenberg ZH, CIC 339, P Zachée (38 (43) 23/15)
Antwerp, Antwerp University College, CIC 996, W Schoeters
(23 (42) 17/16)
Antwerp, AZ Middelheim (hem), CIC 783, R de Boeck (8 (9) 0/8)
Brugge, AZ St Jan (hem), CIC 506, D Selslag, A Van Hoof, J Van
Droogenbroeck, K Van Eygen (50 (59) 23/27)
Brussels, Institut Jules Bordet and the Children’s University Hospital,
CIC 215, D Bron, E Searle, C Devak, K Porfer (38
(43) 28/10)
Brussels, Clinique Universitaires St Luc (hem, onco, peda), CIC 234, A
Ferranti (51 (57) 29/23)
Brussels, Clinique Universitaires St Luc (peda), CIC 234, C Vermylen
(19 (19) 7/3)
Brussels, Cliniques Universitaires St Luc, (onco), JP Machiels (no report)
Brussels, Hôpital Erasme (hem), CIC 596, W Femmans, A Kestens,
M Lambrechts, A De Wever (22 (25) 0/22)
Brussels, University Hospital (hem, onco), CIC 630, B Van Camp,
A Schots (23 (30) 9/17)
Charleroi, Centre Médical, CIC 491, M André
(18 (21) 3/15)
Charleroi, University Hospital (hem, onco), CIC 804, A Treflet
(2 (7) 0/2)
Gent, University Hospital (hem, ads, peda), CIC 744, LA Noens
(51 (55) 23/28)
Haine St Paul, Hôpital de Jolimont (hem), CIC 234, A Delannoy,
C Ravot, N Stramatz (18 (18) 2/16)
Hasselt, Virga Jesse Ziekenhuis (hem), CIC 632, D Vanstraalen,
G Bries, V Mandoe (27 (31) 0/27)
Leuven, University Hospital Gasthuisberg (hem, ads, peda),
CIC 209, J Maertens, MA Boogaerts, P Vandenberghe (88 (97)
56/32)
Liège, CHR, La Citadelle (hem, onco), CIC 353, B De Prijck
(9 (9) 0/9)
Liège, University Hospital Sart-Tilman (hem), CIC 726, Y Bégain
(35 (69) 30/25)
Roere, H Hartziekenhuis (hem, onco), CIC 646, F Van Adts, J
Tytgat, J Denol (16 (10) 4/12)
Wilrijk, Sint Agorina GVA (hem, onco), CIC 715, J Leemans
(6 (6) 0/6)
Yvoir, Clinique universitaire de Mont-Godinne (hem, onco), CIC 234,
C Doyen (21 (26) 3/18)

Bosnia-Herzegovina: (two teams: 6 (6) 1/5)
Sarajevo, Clinical Centre University Sarajevo (hem), CIC 198,
A Sofo-Halajovic (0 (0) 0/0)
Tuzla, University Clinical Centre of Tuzla (hem), CIC 647,
M Malesevic (6 (6) 1/5)

Bulgaria: (two teams: 38 (38) 5/29)
Sofia, Pediatric Hospital for Oncohematology and Bone Marrow
Transplantation (peds-hem-onco), CIC 346, D Bozev, A Avramova,
M Yordanova (20 (20) 9/11)
Sofia, National Centre of Hematology and Transfusiology, BMT,
CIC 859, O Michailov (18 (18) 0/18)

Brazil: (two teams: 32 (33) 6/26)
Zagreb, Clinic Hospital ‘Merkur’, CIC 159, B Jakišić, H Minog
(32 (33) 6/26)
Zagreb, Clinical Hospital Center, CIC 302, B Labar, D Nemet,
M Miric (no report)

Cyprus: (one team: 8 (8) 0/8)
Nicosia Makarios Hospital Ill (hem), CIC 575, A Papastyphonos
(8 (8) 0/8)

Czech Republic: (nine teams: 459 (518) 161/289)
Brno, Masaryk University Hospital (ads, peda, hem, onco), CIC
597, J Voleck, J Mayer, Z Koristek (101 (127) 24/77)
Hradec Králové, Charles University (hem), CIC 729, S Filip,
M Blaha (48 (57) 20/28)
Olomouc, University Hospital (hem, onco), CIC 574, K Indriks
(50 (56) 16/34)
Plzen, Faculty Hospital (hem, onco), CIC 718, V Koza (73 (80)
31/42)
Prague, Clinical Haematology, Charles University, CIC 318,
T Koza (41 (44) 0/41)
Prague, Thomayer Memorial Hospital, CIC 375, J Abrahamova,
J Nepousuk (3 (3) 0/3)
Prague, University Hospital Motol (peds, onco, hem, onco), CIC
6562, P Sedlacek (30 (32) 26/6)
Prague, Institute of Hematology and Blood Transfusion, A Vitek,
P Kolycka CIC 6561 (51 (55) 44/7)
Prague, Charles University, CIC 745, M Trenky (53 (64) 0/53)

Denmark: (four teams: 248 (276) 92/156)
Aalborg, Aalborg Hospital (hematology-immunology), CIC 848,
B Pedersen, J Christiansen (18 (19) 0/18)
Aarhus, Aarhus University Hospital (hem, onco), CIC 634-4-510,
E Segel, B Moeller (50 (54) 0/50)
Copenhagen, Rigshospitalet (hem), CIC 206, N Jacobsen
(152 (173) 92/60)
Copenhagen, Herlev Hospital (hem), University, CIC 568, B Jensen
(28 (30) 0/20)

Estonia: (two teams: 33 (33) 10/23)
Tallinn, North Estonian Regional Hospital, K Vah (12 (12) 0/12)
Tartu, University Hospital (hem, onco), CIC 746, H Everaus,
A Kaare (21 (21) 10/11)
Montpellier, CHU de Montpellier Hôpital Arnaud de Villeneuve, F Bernard (17 (17) 10/7)
Montpellier, CHR Lapeyronie (hem), CIC 926, JF Rossi (139 (166) 48/91)
Mulhouse, Hôpital du Haussieux, B Drieu, M Ojeda (10 (10) 0/10)
Nancy, Vandoeuvre-Ies-Nancy, Hôpital d’Enfants, P Bordignon (46 (51) 38/8)
Nancy, Vandoeuvre-Ies-Nancy, CHU Nancy-Brabois (hem), P Ledetin, P Witz (48 (48) 0/46)
Nantes, Hotel Dieu (hem), CIC 253, P Chevallier, JL Harousseau (194 (195) 67/17)
Nico, Hôpital de l’Arche (including Hôpital Leval), (peds), CIC 523, N Gratteco, JP Castutto, D de Ricard (46 (46) 26/16)
Nico, Centre Antoine Lacassagne, A Thyss (19 (19) 0/19)
Paris, Hôpital Necker (ads, hem), CIC 160, B Varet, C Bellevanger, A Veil (73 (83) 37/42)
Paris, Hôpital Necker des enfants malades (allo), CIC 201, A Fischer (29 (26) 28/1)
Paris, Hôpital St Louis (hem, allo, ads, peds), CIC 207+CIC 748, G Socié, E Glucksch, H Esperou (106 (109) 105/1)
Paris, Hôpital St Louis (auto), CIC 805, G Gissiblrecht (55 (55) 1/54)
Paris, Hôpital St Louis (auto-look), CIC 960, H Decrobert, L Degas, P Roussot (4 (4) 0/4)
Paris, Hôpital St Louis (auto immuno-Haemo), J-P Fermand (38 (38) 0/38)
Paris, Hôpital St Antoine (hem), CIC 213, C Gorin, L Fouillad (43 (53) 12/31)
Paris, Hôpital Enfants Armand-Trousseau, CIC 213, G Leverger, A Aubignyon, L Douzy (10 (10) 0/10)
Paris, Hôpital Dieu (hem), CIC 222, Z Marjanovic (74 (80) 38/36)
Paris, Hôpital Pitie Salpetriere (hem), CIC 262, J-P Vernaut, V Lebland, N Dechini (66 (104) 43/53)
Paris, Institut Curie (ads/onco/peds), CIC 702, J Michon (30 (30) 0/30)
Paris, Hôpital Tenon (onco), CIC 747, JP Lotz (23 (46) 0/23)
Paris, Hôpital Robert Debré, K Yakoubou, A Baroelch (24 (24) 25/0)
Paris, Hôpital Européen GP, JM Andreu, C Le Maigrier (3 (3) 0/3)
Paris, Hôpital d’Instruction des Armées Percy, Cmattet, T de Revel, G Nedelec (47 (55) 26/23)
Paris, Hôpital Cochin (auto), F Dreyfus, M Quarré (35 (38) 0/35)
Pessac, Hôpital Haut-Lévêque, CHU Bordeaux, CIC 267, N Mépiard, G Marab, T Tabrizi (163 (198) 67/58)
Poitiers, Hôpital la Milétrie, CIC 254, M Renaud (80 (87) 45/35)
Poitou, Hôpital René Dubuis (hem, onco), CIC 964, H Gonzalez (14 (19) 0/14)
Reims, Hôpital Robert Debré (hem, onco), CIC 959, A Delmer, B Pignon, C Hembrinck (40 (48) 0/40)
Rennes, CHRU, Clinique Médical Infantil, E Le Gall, V Gardet (9 (10) 5/4)
Rennes, Hôpital de Pontchaillou (hem), T Lamy (82 (85) 26/56)
Roubaix, Hôpital V. Provo (hem), I Plantier-Coquerel (12 (17) 0/12)
Rouen, Centre Henri Becquerel, CIC 941, H Tilly, P Lenain (68 (79) 17/51)
Rouen, Hôpital Charles Nicolle, JP Vannier (12 (14) 6/6)
St Cloud, Centre René Huguenin, CIC 351, M Janvier (28 (29) 0/28)
Strasbourg, Hôpital Hautepierre, B Lionne (82 (101) 30/52)
Strasbourg, Hospices Civils, Service de Pédiatrie 5, P Lutz (13 (18) 9/46)
Toulouse, Hôpital de Purpan (hem), CIC 624, M Attal, J-C Nogaro (13 (13) 4/9)
Toulouse, Hôpital de Purpan (peds), CIC 624, H Rubie (112 (122) 29/83)
Tour, Hôpital Bretonneau (onco), CIC 272, P Colombat (50 (57)
0/50)

Valenciennes, Hosp. De Valenciennes, M Simon (19 (19) 0/19)
Villejuif, Institut G Roussy (pedi), CIC 503, O Hartmann,
D Valloz-Couanet (72 (94) 0/72)
Villejuif, Institut G Roussy (ads, hem), CIC 666, J-H Bourhis,
C Boschacq, J-M Valetton (150 (150) 40/110)
Villejuif, Hôpital Paul Broussais, B Dolmas-Marsalet (3 (5) 0/3)

Georgia: no report

Germany: (108 teams: 4619 (5856) 2049/2570)

Aachen, Universitätshäklinikum RWTH (hem, onco), Med Klinik IV,
CIC 348, A Giebeler, G Gebauer (14 (21) 0/14)

Augsburg, Zentralklinikum (hem, onco), Med Klinik II,
G Schimmik, M Sandhein (32 (73) 16/16)

Bad Saarow, Humaine Klinikum, G Schultz, U Wreck,
K Sendtner (13 (24) 0/13)

Berlin, Universitätsklinik der FU Charité Campus Virchow
Klinikum (pedi), CIC 356, G Schneider, W Ebel, J Knüll (32 (39) 24/28)

Berlin, Universitätsklinik der FU Charité Campus Virchow
Klinikum (ads, hem, onco), CIC 807, B Dörken, R Arnold (93 (116)
53/40)

Berlin, HELIOS Klinikum Berlin, Robert-Röske Klinik (hem, onco),
CIC 518, W-D Ludwig, R Bargan (26 (37) 0/26)
Berlin, Universitäts-Klinik der FU Benjamin Franklin (hem, onco),
CIC 590, L Ulrich, A Thiad (71 (92) 39/2)

Bielefeld, Frankfurter Krankenhaus (hem, onco), HJ Wehr, A Zunz
Spreckel (5 (5) 0/5)

Bochum, Krankenhaus der Erde (hem, onco), CIC 124,
W Schmitz, C Tesche (21 (37) 35/30)

Bohn, Rheinische Friedrich-Wilhelms-Universität (ads, hem, onco),
T Sauerer, I Schmidt-Wolf (21 (36) 0/21)

Bonn, Rheinische Friedrich-Wilhelms-Universität (pedi, hem, onco),
U Bode, C Haan (8 (14) 0/8)

Braunschweig, Städtisches Klinikum (hem, onco), CIC 674,
B Wöllmann, T Galeyk (15 (20) 0/15)

Bremer, Zentralklinikum St. Jürgenstrasse, CIC 602, B Hertzen-
stein, H Rasche, H Thomsen (23 (38) 7/16)

Brezen, DIAKO (hem, onco), KF Pflüger, T Wolff (15 (20) 0/15)

Chemnitz, Krankenhaus Köschwalt (hem, onco), CIC 104, M Härd,
G Isserlin (46 (70) 14/35)

Cottbus, Carl-Thiem Klinikum, Med Klinik II (hem), H Steinhauser,
N Peter (21 (33) 0/21)

Dassau, Städtisches Klinikum Dassau (hem, onco), M Plauth,
A Florschütz (13 (18) 0/13)

Dortmund, St Johannes Hospital (hem, onco), H Pfeifer,
M Hindelg (7 (9) 0/7)

Dresden, Universitätshäklinik Carl Gustav Carus (hem, onco),
CIC 508, G Ihminger, M Bormhäuser (155 (177) 95/60)

Duisburg, St Johannes Hospital, CIC 519, C Aul, R Hartwig (23
(33) 0/23)

Düsseldorf, Heinrich-Heine Universität, Medizinische Klinik (hem,
onco) and St Antonius Hospital, Eschweiler (hem, onco), CIC 390,
R Haas, G Kobbe, R Fuchs (95 (115) 148/47)

Dültschendorf, Heinrich-Heine Universität, Zentrum für Kinderheilkun-
de, CIC 651, U Giselh, D Dillor (33 (39) 19/4)

Erlangen, Universität Erlangen-Nürnberg (hem, onco), Med
Klinikum II, CIC 809-1, R Röder (34 (39) 18/6)

Erlangen, Universität Erlangen-Nürnberg (hem, onco), Med
Klinikum II, CIC 809-2, W Holzer (15 (17) 11/4)

Essen, Universitätshäklinik (ads, pedi), CIC 259, DW Beeken,
R Peceny, W Favers, K Brenna, O Basu (178 (190) 165/15)

Essen, Evangelisches Krankenhaus Essen-Werden GmbH (hem,
onco), CIC 784, W Heit, M Wiatrad (59 (64) 16/43)

Essen, Universitätshäklinik (hem), C Dähkén, R Noppenny (19
(26) 0/19)

Eisen, West German Cancer Center, S Seedor, T Moritz (27 (61)
0/27)

Frankfurt, KH Nordwest, A Kauth, E Jäger (6 (6) 0/6)
Frankfurt, Klinikum Frankfurt (Oder), CIC 190, M Kiehl (10 (23)
0/10)
Frankfurt a. M., Universitätshäklinikum d. JG Goethe (hem, onco,
pedi), CIC 138, T Klingebiel (25 (28) 18/77)
Frankfurt a. M., JG Goethe-Universität (ads), CIC 297, D Hoerber,
H Martin (72 (87) 32/40)
Frankfurt/Mainz, Städtisches Krankenhaus (ads), H Berische,
H Flock (9 (11) 0/9)
Frankfurt/Mainz, Onkologische Gemeinschaftspraxis, CIC 193,
W Knuf (8 (12) 0/8)

Freiburg i. Br., Universitätshäklinik (ads, hem, onco), Med Klinik I,
CIC 810, R Mentelmann, J Junke, M Engelhardt (167 (188) 95/72)
Freiburg i. Br., Universitätshäklinik (hem, onco), CIC 810, C
Niemeyer, U Düffter (23 (35) 18/9)

Gießen, Universitätshäklinik (hem, onco), CIC 326, A Reiter,
W Wössmann (13 (14) 9/4)

Göttingen, Georg-August Universität (hem, onco), CIC 552,
L Trümper, B Hiss (79 (93) 38/41)

Grenzland, Erna-Mörth-Arztliche Universität (ads + pedi), CIC 530,
C Dölken, W Krüger (33 (42) 17/11)

Gütersloh, Städtisches Krankenhaus (hem, onco), C Gropp,
S Rösel (3 (5) 0/3)

Hagen, Kath. Krankenhaus (hem, onco), CIC 536, H Eimermann,
W Liedemann (19 (25) 0/19)

Hall, Martin Luther Universität (hem, onco, ads), CIC 338,
G Behre, H-J Schmidt, M Christopeit (95 (75) 23/36)

Halle, Martin Luther Universität (hem, onco, pedi), CIC 654,
G Hornell, J Fölö (4 (4) 2/4)

Hamberg, KH St George (hem, onco), CIC 153, N Schmidt,
P Dreger (36 (63) 5/31)

Hamberg, Allgemeines Krankenhaus Altona (hem, onco), CIC 366,
D Brausmann, H Salwender (43 (69) 9/43)

Hamberg, Eppendorf-Krankenhaus (hem, onco, pedi), CIC 614,
AR Zander, N Krüger (137 (154) 117/20)

Hamberg, Eppendorf-Krankenhaus (hem, onco, ads), Med Klin II,
CIC 573, C Bokemeyer (36 (65) 0/36)

Hannover, Kreiskrankenhaus Hanam (hem, onco), H Schmidt,
K Bohrmann (11 (20) 1/10)

Hann, St Marien Hospital (hem, onco), H Dörk, H Pelz (8 (12)
0/8)

Hann, Evangelisches Krankenhaus (hem, onco), CIC 509,
L Balesius (30 (30) 0/30)

Hannover, Medizinische Hochschule (hem, onco, ads), CIC 295,
A Gunter, C Hartenstein (53 (103) 57/26)

Hannover, Medizinische Hochschule (hem, onco, pedi), CIC 295,
K Wiek, K Sykora (26 (29) 20/6)

Hannover, KH Siloah, CIC 342, H Kirschner, M Sosada (16 (16)
0/16)

Heidelberg, Ruprecht-Karls-Universität-Poiklinik (hem, onco),
CIC 524, P Deger, AD Ho, U Hegener (133 (327) 15/16)

Hohenzollern, Universität des Saarlandes (hem, onco), CIC 785,
M Pfeimmelsdibl, J Schuhet (69 (96) 18/51)

Kaiserslautern, Westpfalz Klinikum (hem, onco), CIC 357, H Link,
F-G Hagemann (7 (9) 0/7)

Karlsruhe, Städtisches Klinikum (hem, onco), CIC 290, M Bents,
S Wüllgen (73 (37) 0/23)

Kassel, Städtische Kliniken (hem, onco), M Wolf, E Steinhauser
(11 (14) 0/11)
Shiraz, Nemazee Hospital (hem, onco), CIC 188, M Ramzi (40 (40)
22/18)

Teheran, Shariat Hospital (hem, onco), CIC 633, A Ghavamzadeh
(324 (325) 214/116)

Israel:

Beit Shean, Hadassah University Hospital (hem, onco), CIC 245, J Rowe
(171 (172) 35/35)

Jaffa, Rambam Medical Center (hem, onco, Peds), CIC 764, O Sigal
(42 (43) 17/17)

Jaffa, Sackler School of Medicine, CIC 273, G Kassal (40 (40)
13/13)

Jaffa, Sackler School of Medicine, CIC 273, G Kassal (40 (40)
13/13)

Jaffa, Sackler School of Medicine, CIC 273, G Kassal (40 (40)
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Jaffa, Sackler School of Medicine, CIC 273, G Kassal (40 (40)
13/13)

Jaffa, Sackler School of Medicine, CIC 273, G Kassal (40 (40)
13/13)
Padova, Instituto Oncologia Veneto (IVO), Oncologia Medica II, CIC 319, S Averna, S Montefusco (10 (12) 0/10)
Palermo, Ospedale di Bambini (pediatri, orco), CIC 109, D Ceselli (15 (15) 5/10)
Palermo, Ospedale V Cervello (hem), CIC 392, R Scimà, A Cavallaro (48 (58) 21/27)
Palermo, Ospedale La Maddalena (hem, orco), CIC 592, M Mastro, F Porretto, A Crescimanno (56 (67) 14/42)
Palermo, Div. di Ematologia con Trapianto di Midullo, Uni degli studi di Palermo (hem), CIC 814, E Iannitto (9 (12) 0/9)
Parma, Cattedra di Ematologia, Università di Parma, CIC 245, V Rizzoli, M Mangione (17 (21) 1/16)
Pavia, Policlinico S Matteo (hem), CIC 286, EP Alessandrino (84 (85) 25/59)
Pavia, Policlinico S Matteo (hem, orco, pedi), CIC 557, F Locatelli (102 (103) 9/9)
Pavia, Policlinico S Matteo (orco), CIC 562, M Danova (90 (90) 0/0)
Pavia, Fondazione S Maugeri (orco), CIC 771, A Zambelli, G Robustelli della Cuna (12 (14) 11/11)
Perugia, Policlinico Monteluce (orco), CIC 573, AM Libranzi, F Grigsoni (5 (9) 0/5)
Perugia, Policlinico Monteluce (orco), Università, CIC 794, MF Martelli, F Azzini, A Taboilo (111 (116) 52/59)
Pescara, Ospedale San Salvatore, CIC 529, G Visani, G Locatelli (32 (39) 16/16)
Pescara, Ospedale Civile (hem), CIC 248, P di Bartolomeo (50 (54) 40/10)
Pescara, Pisanza, Ospedale Civile (hem, orco), CIC 163, L Cavanna (20 (24) 3/13)
Pisa, University of Pisa (pedi, hem, orco), CIC 795, C Faver (21 (22) 14/7)
Pisa, University of Pisa (ada, hem, orco), CIC 132, M Petroni, F Papinetti (57 (72) 16/43)
Ravenna, Ospedale Civile (hem, orco), CIC 306, G Rosi (56 (43) 0/36)
Reggio di Calabria, Azienda Ospedaliera di Reggio Calabria, CIC 587, P Ispodino, G Consoli (83 (98) 26/57)
Reggio Calabria, Arcispedale S Maria Nuova (hem), CIC 660, L Gugliotta (24 (27) 8/16)
Rimini, Ospedale Infermi Rimini (hem, orco), P Fattori (15 (18) 0/15)
Rionero in Vulture, Centro di Riferimento Oncologico della Basilicata (hem), CIC 185, P Musto, N Di Ronza (5 (6) 1/4)
Roma, Università "La Sapienza" (hem), Faculty I, CIC 232, R Foa, G Meloni (107 (128) 26/81)
Roma, Ospedale S Camillo (hem), CIC 287, I Majolini, A Locatelli (41 (42) 19/22)
Roma, Università Cattolica (hem), CIC 307, S Cuore, S Sica, G Leone (59 (68) 19/40)
Roma, Universitario Tor Vergata (hem) CIC 755, Ospedale Bambino Gesù (hem), Regina Elena Cancer Institution (hem, orco), Università "La Sapienza" (hem) Faculty II, W Arrigo, S Adrando, P De Fabritiis, G De Rossi, MC Pettis, G Avisiani, B Monarca, L Annino (122 (146) 47/59)
Roma, Ospedale Bambino Gesù (orco), CIC 796, A Donnfrancesco, A Jenkner, A Castellano, L De Sio, R Cozza, P Fidani, G De Laurentiis (33 (29) 0/23)
San Giovanni Rotondo, Hospital Casa Sollievo Sofferenza (hem), CIC 526, N Cascavilla, M Corsetti, G Greco (50 (65) 20/30)
Sassari, Universita Di Sassari (hem) CIC 870, M Longinotti (8 (10) 0/8)
Siena, Ospedale Scolavo (hem), CIC 321, F Lauria (29 (37) 9/20)
Taranto, Ospedale Nord (hem), CIC 332, P Maza, G Alboni, D Amurri (46 (50) 13/33)
Torino, Ospedale Pediatrico S Giovanni, CIC 231, M Falda, F Locatelli (76 (99) 35/14)
Torino, Ospedale Regina Margherita (pedi), CIC 305, F Fagiolini, E Valetti (80 (52) 20/20)
Torino, Ospedale Mauriziano Umberto 1, IRCC, CIC 377, M Agliotta, A Capaldi, P Carecchio (18 (24) 10/8)
Torino, Ospedale S Giovanni (hem), CIC 696, M Boccadoro, M Massaia, C Tarello, B Benedetto, D Caracciolo, A Fileri (77 (128) 15/62)
Trieste, Logistic Hospital C Panico, CIC 652, V Pavone (22 (25) 3/3)
Trieste, Istituto per l'Infanzia, Clinical Pediatrica, CIC 252, M A洮ion (23 (23) 15/18)
Udine, Policlinico Universitario (hem), CIC 705, R Farnini (79 (94) 37/42)
Venecia, Ospedale Civile Riuniti di Venezia (hem), CIC 502, T Chiotti, M Vespignani, M Stimato (17 (23) 3/4)
Verbania-Pallanza, IUIA Oncologia Medica, Ospedale di Verbania, CIC 385, A Lunazzi (8 (8) 0/8)
Verona, Policlinico di Bologna (hem, orco), CIC 623, C Gerlatti, G Pieroni, F Benedetti, G Cetto (55 (75) 20/35)
Vicenza, Ospedale S Bortolo (hem), CIC 797, R Raimondi, F Rodighiero (50 (59) 15/35)
Latvia: (one team): 14 (14) 3/11
Riga, Clinic Lancers, CIC 583, S Lejnie (14 (14) 3/11)
Lebanon: (one team): Beirut, American University of Beirut, CIC 369, A Bazarbachi
Lichtenstein: no report
Lithuania: (two teams): 90 (110) 33/60
Vilnius, University Hospital Santariskiu Klinikos (hem), CIC 644, A Slobina, I Trockulis (81 (97) 30/51)
Vilnius, University Children's Hospital (hem, orco), CIC 508, J Rassou (12 (13) 3/9)
Luxembourg: no report
Macedonia: (one team): 14 (17) 4/10
Skopje, Medical Faculty (hem), CIC 381, B Georgievski (14 (17) 4/10)
Malta: no report
Moldova: no report
Morocco: no report
Netherlands: (13 teams): 850 (919) 357/503
Amsterdam, Academic Medical Center (ads, pedi), CIC 247, J van der Lee, H van den Berg (pedi) (44 (52) 14/30)
Amsterdam, Free University Hospital (hem), CIC 588, GJ Oosterkophke (97 (104) 43/54)
Amsterdam, The Netherlands Cancer Institute, CIC 976, S Redelhi, J Baars (26 (39) 0/26)
Enschede, The Medisch Spectron Twente, CIC 361, Dr Schaaften (15 (15) 0/15)
Groningen, University Hospital (hem), CIC 546, G van Houth (66 (70) 6/60)
The Hague, Haga Hospital (Leiden), CIC 547, PW Wijermans (26 (26) 0/26)
Leiden, University Medical Center (ads, pedi), CIC 203, R Wilkeme, M Egelar (95 (105) 76/23)
Maastricht, University Hospital (hem, orco), CIC 565, H Schouten, J Wagstaff (73 (78) 19/54)
Nieuwegein, St Antonius Hospital, CIC 200, D Biesma, G Veth, O de Weerd (18 (20) 0/18)

Bone Marrow Transplantation
Nijmegen, University Hospital (ads, pols, onco), CIC 237, A Schattenberg, P Hoogerbrugge (105 (12) 51/54)
Rotterdam, Dr Daniel den Hoed Cancer Center, CIC 246, P.J Cornelissen (149 (151) 59/90)
Utrecht, University Hospital (hem, ads, pols), CIC 239, LF Verdonck, NM Wolfsraat (130 (135) 89/41)
Zwolle, Isaas Klinieken/Sophia Ziekenhuis, CIC 548, M von Marwijk Kooi (12 (12) 0/12)

Norway: (six teams: 205 (217) 62/143)
Borgen, Haukeland Universitetets Sjukehus, CIC 197, F. Ernst (31 (35) 6/25)
Oslo, Rikshospitalet Radiumhospital, CIC 235, D. Albrechsen, L. Brinch (75 (78) 53/23)
Oslo, Rikshospitalet Radiumhospital (onco), CIC 782, G. Lautersten, S. Kvalvo (41 (46) 3/28)
Oslo, Ulleval Universitetets Sykehus (hem), F. Woldoff, J-M Tangen (29 (29) 0/29)
Tromsø, University Hospital of Northern Norway (hem), IM Dahl (8 (8) 0/8)
Trondheim, St Olavs Hospital, J. Hammerstrom, A. Waage (21 (21) 0/21)

Poland: (17 teams: 774 (866) 281/493)
Bydgoszcz, Nicolaie Copernicus University (peds, hem, onco), CIC 764, M. Wysocki, J. Sytnicki (20 (22) 5/15)
Gdansk, Medical University (hem), CIC 799, A. Hellmann (54 (54) 17/37)
Katowice, Silesian Medical Academy (hem), CIC 677, J. Holowicki (126 (127) 57/79)
Krakow, Jagiellonian University (hem), CIC 553, A. Skotnicki (43 (49) 6/37)
Krakow, Polish-American Children's Hospital, JUMC, CIC 507, J. Goralnick (8 (8) 4/8)
Lodz, Medical University of Lodz (hem), CIC 171, T. Robak (20 (21) 0/20)
Lublin, Children’s University Hospital (hem, onco), CIC 678, J. Kowalczyk (16 (16) 11/5)
Lublin, University Medical School (hem, onco), CIC 695, A. Demyszynska, W. Wach, A. Walter-Çonek, W. Legiec (54 (55) 5/49)
Poznan, Institute of Pediatrics, CIC 641, J. Wachowiak (28 (28) 23/27)
Poznan, K. Marcinkowski University (hem), CIC 730, M. Komarnicki (68 (69) 23/45)
Warsaw, Institute of Hematology and Blood Transfusion, CIC 693, B. Marianska, L. Konopka, B. Naslowski, K. Halabarda, M. Szczepiak (28 (30) 17/11)
Warsaw, Maria Sklodowska-Curie, Centre of Oncology, CIC 800, J. Walewska (59 (61) 0/59)
Warsaw, Central Hospital Military Medical Academy (hem, onco), CIC 816, P. Rzepecki, K. Solnicki, C. Szczyluk (34 (43) 8/36)
Warsaw, Central Clinical Hospital (hem), CIC 954, W. Wiktorski-Żejdzejek, A. Dębala, M. Rokicka (59 (80) 16/43)
Wrocław, Lower Silesian Centre for Cellular Transplantation with National Bone Marrow Donor Registry, CIC 538, A. Langer (52 (61) 28/24)
Wrocław, Medical Academy (hem), CIC 699, K. Kuliński (29 (29) 9/29)
Wrocław, University of Medicine (peds, hem, onco), CIC 817, A. Chybicka (66 (72) 52/14)

Portugal: (six teams: 285 (331) 88/197)
Coimbra, University Hospital, CIC 164, N Costa (23 (25) 0/23)
Lisbon, Instituto Portugues de Oncologia, CIC 300, M. Abecasis, F. Leal Costa (65 (70) 25/60)
Lisbon, Hospital de Santa Maria, CIC 536, J. Alves de Carvalho, F. de Lacerda (34 (40) 16/18)
Lisbon, Hospital de St Antonio dos Capuchos, CIC 826, A. Botelho de Sousa (42 (42) 0/42)
Porto, Instituto Portugues de Oncologia, CIC 291, F. Fintel, F. Campillo (94 (102) 47/47)
Porto, Hospital S João (hem, onco), CIC 329 (merged with CIC 572), J. Guimarães, P. Figueira (27 (40) 0/27)

Romania: (three teams: 52 (58) 9/43)
Bucharest, Padieni University Hospital (hem), CIC 296, AD Moisian, D. Costea, C. Anton (28 (30) 3/20)
Targu-Mures, Seraf Clinic of Hematology, CIC 178, I. Benea (21 (22) 4/17)
Timisoara, University of Medicine (III peds, hem/onco), CIC 174, M. Serban, C. Ionescu (6 (8) 0/6)

Russia: (17 teams: 333 (358) 112/221)
Ekaterinburg, City Hospital no. 7, AB Logino (no report)
Ekaterinburg, Regional Hospital no. 1, KS Kostantinov, VA Shantar (25 (25) 8/58)
Moscow, Russian Children’s Hospital (hem), CIC 694, A. Maslen, E. Skorobogatko, P. Pashkov (43 (50) 35/58)
Moscow, Cancer Research Center, CIC 757, KN Melkov (35 (35) 1/34)
Moscow, Institute of Biophysics, AE Baranov (10 (10) 0/10)
Moscow, Cancer Research Center (peds, hem/onco), G. Mentreich (27 (27) 8/19)
Moscow, Research Hematology Center of RAS, VG Savchenko (35 (39) 20/15)
Moscow, Main Military Clinical Hospital (hem), SV Shamasky (23 (27) 0/23)
Moscow, City Clinical Hospital no. 38, NA Obidina (0 (0) 0/0)
Novosibirsk, Institute of Clinical Immunology, CIC 576, I. Lisyukov (45 (45) 0/45)
Samara, Regional Hospital, VA Rost (28 (30) 8/28)
St Petersburg, Clinical Center for Advanced Medical Tech, CIC 370, E Podolsteva, V. Soldatenkov, O. Ryazanskaya (no report)
St Petersburg, Military Medical Academy (hem), CIC 520, A. Novik (no report)
St Petersburg, Research Institute of Hematology, KM Abdulladirov (14 (14) 0/14)
St Petersburg, State Pavlov Medical University (hem), CIC 725, BV Afanasiev, L. Zubarovskaya (55 (55) 42/13)
St Petersburg, Leningrad Regional Clinical Hospital, IS Zuyzgin (7 (7) 0/7)
Yaroslavl, Regional Clinical Hospital (hem), VA Lapin (8 (8) 0/8)

Sao Marino: no report

Saudi Arabia: (two teams: 218 (232) 153/65)
Riyadh, King Faisal Specialist Hospital and Research Centre (onco, ads, hem), CIC 397.1, M. Al Jarfi (113 (118) 62/54)
Riyadh, King Faisal Specialist Hospital and Research Centre (peds, hem, onco), CIC 397.2, M. Ayes (105 (114) 91/14)

Serbia and Montenegro: (four teams: 59 (67) 18/41)
Belgrade, Mother and Child Health Institute, CIC 358, D. Vujic (12 (12) 0/6)
Belgrade, Clinical Centre of Serbia (hem), CIC 373, J. Bota, D. Antić (10 (10) 0/10)
Belgrade, Military Medical Academy (hem), CIC 582, D. Stanićov (29 (36) 12/17)
Novi Sad, Institute of Internal Diseases, Clinical Centre of Novi Sad (hem), CIC 655, S. Popović (8 (8) 0/8)

Slovakia: (five teams: 144 (150) 31/113)

Bone Marrow Transplantation
Banze Bystrica, Roosevelt Hospital (hem), CIC 333, I Markuljak, F. Kralj (14 (15) 6/14)
Bratislava, National Cancer Institute, CIC 560, J Lakota (70 (70) 6/64)
Bratislava, University Hospital (hem), CIC 610, M Mistrik (31 (35) 17/14)
Bratislava, University Hospital, 2nd Children’s Clinic, CIC 684, S Sutiarova, J Horakova, I Bodon (11 (13) 8/5)
Kosice, University Hospital LF UP JS (hem), CIC 984, E Totohova (18 (18) 0/18)
Slovenia: (one team): 75 (85) 18/57
Ljubljana, University Medical Centre (hem), CIC 640, I Pretnar (75 (85) 18/27)
South Africa: (one team)
Cape Town, Constantiaberg Medi Clinic (hem), CIC 772, P Jacobs, I Wood
Spain: (65 teams): 1803 (1927) 59/1208
Albacete, Hospital General, C Rivas-Gonzalez (19 (19) 6/19)
Barcelona, Hospital Clinic (hem, onco), CIC 214, E Montserrat, E Carreiras (90 (93) 50/64)
Barcelona, Santa Creu i Sant Pau (adults), CIC 260, J Sierra, S Brunet (94 (103) 48/6)
Barcelona, Santa Creu i Sant Pau (ped), CIC 260, I Badell Serra, J Cubells-Rierro (11 (13) 4/7)
Barcelona, Hospital Vall d’Hebron, Materno Infantil, CIC 527-1, J Sanchez de Toledo Godina (45 (46) 30/13)
Barcelona, Hospital General Vall d’Hebron, CIC 527-2, A Julia, Font, J Zuazua (24 (25) 6/18)
Barcelona, Hospital Mutua de Terrassa (hem-onco), T Marti (10 (10) 0/10)
Barcelona, Hospital Universitario Germans Trias i Pujol, CIC 613, J Ribera (53 (53) 18/35)
Barcelona, Hospital Sant Joan de Deu, CIC 668, J Estella Aguado (5 (5) 0/8)
Barcelona, Hospital Duran i Reynals (hem), Institut Catala d’Oncologia, CIC 779, R Duarte Palomino, C Ferrer, J Berlanga, A Fernandez (31 (34) 16/15)
Caceres, Hospital San Pedro de Alcastar, M Luz Amigo Lozano (10 (11) 0/10)
Cadiz, Hospital del SAs de Jerez (hem), CIC 612, A Leon (24 (27) 7/17)
Cadiz, Hospital Universitario ‘Puerta del Mar’ (hem), CIC 679, J Gil (5 (5) 0/5)
Canary Isles, Las Palmas, Hospital Insular (hem), CIC 335, J Gonzalez-San Miguel (7 (8) 0/7)
Canary Isles, Las Palmas, Hospital Materno-Infantil (hem, onco), J Lodos Rojas, A Molinés (3 (3) 0/3)
Canary Isles, Garaf, Hospital Universitario de Gran Canaria ‘Dr Negrin’, CIC 537, T Madero, R Matia, C Campo, J Jimenez (17 (17) 5/12)
Canary Isles, Tenerife, Hospital Universitario de Canarias, J Hernandez Nieto, MT Hernandez Garcia (22 (22) 0/22)
Canary Isles, Tenerife, Hospital Ns De La Candelaria, J Garcia-Talavera, J Beris, P Rios Bull (5 (5) 0/8)
Castello de La Plana, Hospital General de Castellon (hem), R Garcia-Bayo (8 (8) 0/8)
Cordoba, Hospital Reina Sofia (hem), CIC 238, A Torres Gomez (57 (62) 22/25)
Cruces-Barakaldo, Hospital de Cruces (hem), CIC 393, J Zazca Verde, F F assortment (30 (36) 0/30)
Galicia, Hospital de Galicia (hem), CIC 975, J Qures, K Atxa (16 (16) 0/16)
Granada, Hospital Virgen de las Nieves (hem), CIC 559, JM de Pablos Gallego (24 (28) 6/20)
Jaen, Hospital Ciudad de Jaen (hem), A Alcalan (11 (11) 0/11)
La Coruña, Complejo Hospitalario Juan Canalejo, CIC 361, FJ Baillale, C Ramirez, P Torres, R Rodriguez, R Vazquez (38 (41) 4/34)
Lleida, Hospital Aran de Villanueva, J Macia (5 (5) 0/5)
Lugo, Hospital Xeral-Cakle, M Gonzalez-Lopez (7 (7) 0/7)
Madrid, Hospital de la Princesa, CIC 236, JM Fernandez Ramirez, A Figuera, A Alegre (45 (51) 28/17)
Madrid, Hospital Doce de Octubre, CIC 382, JJ Labuerta (hem)
II Cortes Funes (onco), J Lopez Perez (ped) (64 (66) 10/54)
Madrid, Hospital Ramon y Cajal (adults), CIC 615, J Glez Polo, J Perez de Urtica, J Lopez, J Garcia Larrea (30 (30) 8/22)
Madrid, Hospital Ramon y Cajal (ped), CIC 615, A Munoz Villa (4 (4) 3/1)
Madrid, Clinica Puerta de Hierro (hem), CIC 728, MN Fernandez, JR Cabrera Marin (22 (28) 12/10)
Madrid, Hospital Nino Jesus (ped, onco), CIC 732, MA Diaz (39 (48) 30/9)
Madrid, Hospital Universitario San Carlos, CIC 733, J Dizze Meidavipilla, I Llorente, R Martinez (28 (28) 0/28)
Madrid, Hospital La Paz Infantil (hem, onco) and Hospital General La Paz (adults), CIC 734, A Martinez-Rubio, A Sastre, F Hernandez Navarro, M Canalejo (43 (46) 25/18)
Madrid, Unidad de TMO-ONC 4, Hospital Gregorio Maranon, CIC 199, JL Diz de Martin (40 (45) 19/21)
Madrid, Clinica Moncloa (hem, onco), JM Fernandez-Ramada, A Escudero (13 (13) 0/13)
Madrid, Clinica Ruber, JM Fernandez-Ramada, A Escuder (17 (17) 0/17)
Madrid, Hospital Universitario de Getafe (hem), F Owen, Compan, N Somolinos (7 (8) 0/7)
Madrid, Fundacion Jimenez Diaz (hem, onco), CIC 309, JL Lopez Lorenzo, F Lebo, M Callejas (15 (15) 0/15)
Madaga, Carlos Haya Hospital (hem), CIC 576, M Gonzalez, M Pascual (55 (55) 15/40)
Murcia, Hospital Univ. Virgen de la Arrixaca, CIC 323, A Morales-Lazaro, MJ Majado-Martinez (21 (22) 0/21)
Murcia, Hospital Morales Meseguer, CIC 735, JM Moralera Jimenez, V Vicente-Garcia, I Heras (48 (53) 24/24)
Oviedo, Hospital Cristal-Pajari (hem, onco), J-L Sestero-Moral (12 (15) 0/12)
Oviedo, Hospital Covadonga (hem), CIC 642, D Carrera Fernandez (26 (26) 11/15)
Palma de Mallorca, Hospital Son Dureta (hem), CIC 722, J Berlith, M Canario (25 (34) 11/14)
Palma de Mallorca, Hospital Son Llatzer, CIC 110, J Bargay-Llarrut (14 (15) 0/14)
Pamplona, Hospital de Navarra (hem), CIC 577, M Orue, MJ Uziz (24 (24) 0/24)
Pamplona, Clinica Universitaria de Navarra, CIC 737, J Rifa (34 (36) 8/26)
Posteveda, Hospital Montecelo (onco), CIC 549, M Cesteta (11 (11) 0/11)
Salamanca, Hospital Clinico, CIC 727, D Caballero (83 (83) 31/25)
San Sebastian, Hospital Nuestra Senora de Aranzazu, CIC 598, R Lasa, J Marin, D Martinez (32 (33) 5/27)
Santander, Hospital Universitario M de Valdecilla (hem), CIC 242, A Iriondo, E Codere (52 (56) 22/30)
Santiago de Compostela, Hospital Xeral de Galicia (hem), CIC 570, JL Bello (20 (22) 5/15)
Sevilla, Hospital Universitario Virgen del Rocío, CIC 769, I Espigado (52 (56) 25/27)
Tarragona, Hospital de Tarragona Joan XXIII (hem), A Llorente Cabrera (11 (11) 0/11)
Valencia, Hospital Clinico Universitario (hem, onco), CIC 282, J Garcia-Conde, C Solano (61 (65) 22/59)
Valencia, Hospital Infantil La Fe (pedos, onco), CIC 653, V Castel, A Verdugo, JM Fernandez (27 (27) 11/16)
Valencia, Hospital Universitario La Fe (hem), CIC 663, MA Sanz, GF Sauron (80 (87) 46/54)
Valencia, Hospital Doctor Peset (hem), P Ribas Garcia (8 (6) 0/8)
Valencia, Instituto Valenciano de Oncologia, V Guillen (3 (3) 0/3)
Valladolid, Hospital Rio Hortega, CIC 611, J Garcia Frade (18 (20) 0/18)
Vigo, Hospital Xeral-Cies, A Martinez-Dalmau (25 (27) 4/21)
Zaragoza, Clinico Universitario Lozano Blesa (hem, onco), CIC 511, I Palomera, M Gutierrez, A Tres, J Mayordomo (12 (12) 9/12)
Zaragoza, Hospital Miguel Servet (hem + onco) M Giralt, G Perez-Llagnan, D Rubio-Felix, A Anton (13 (13) 4/9)

Sweden: (eight teams: 555 (604) 198/357)
Goteborg, CHECT (ads + ped), CIC 289, M Brune, A Fasth (112 (124) 38/74)
Linköping, University Hospital (hem), CIC 740, N Theorell (35 (39) 7/28)
Lund, University Hospital (hem), CIC 283, S Lenhoff (90 (94) 53/57)
Malmö, University Hospital, T Ahlgren (7 (7) 0/7)
Örebro, University Hospital (hem, onco), CIC 738, U Tisdall (15 (20) 0/15)
Stockholm (Huddinge), Karolinska University Hospital (hem, onco), CIC 211, P Juhansson (147 (159) 73/74)
Umea, Norland University Hospital, CIC 731, A Wahlin, V Lazarevic, J Linde, B Markvearn (52 (50) 14/38)
Uppsala, University Hospital (ads + ped), CIC 266, G Oberg (97 (105) 35/44)

Switzerland: (nine teams: 314 (427) 127/204)
Aarau, Kantonsospital (hem, onco), CIC 316, M Wernli, M Bargetzi (23 (30) 0/23)
Basel, Kantonsospital (hem, onco), CIC 202, A Gratwohl, T Kühne, R Herrmann (76 (101) 61/15)
Bellinzona, Ospedale San Giovanni (hem, onco), CIC 829, P Cavalli, M Ghelmann, L Leoclini (13 (17) 4/13)
Bern, Inselspital (ads, ped, onco), CIC 221, K Leibundgut, C Zwicki, M Fey, T Fabel (42 (57) 0/42)
Geneva, Hôpital Cantonal Universitaire (hem, onco), CIC 261, J Pasweg, Y Chaldona, P Wacker (34 (38) 34/0)
Lausanne, CHUV (hem, onco), CIC 820, M Schapira, T Kovacsiovics, S Levyza, N Ketteler (52 (65) 0/52)
St Gallen (hem, onco), Kantonsospital, CIC 324, U Hess (13 (18) 0/13)
Zürich, University Hospital (ads, hem, onco), CIC 208, U Schanz, C Renner (70 (82) 27/45)
Zürich, University Hospital (ads, ped, onco), CIC 334, R Seger (19 (19) 15/3)

Tunisia: (one team: 97 (113) 44/53)
Tunis, Centre National de Greffe de Moelle Ossea, CIC 183, B Othman-Tarek (97 (113) 44/53)

Turkey: (26 teams: 790 (823) 394/396)
Adana Yureqir, Baskesti University Adana Research and Training (hem), CIC 589, H Ozdogu (7 (7) 3/4)
Ankara-Sihhiye, Hacettepe University (hem), CIC 168, H Goker, O Onur, F Hanesdaglisk, S Dundar (14 (14) 13/1)
Ankara-Bezost, Gazi University (hem), CIC 169, G Sucuk (56 (69) 35/23)
Ankara, Hacettepe University, Institute of Oncology, CIC 292, S Karsu, Y Koc, E Ozdemir (29 (30) 3/28)
Ankara-Elitik, GATA BMT Center, CIC 372, F Arpaolu, A Özet, C Beyan, A Ural (60 (60) 21/39)

Ankara, Ilhan Doganacmi Childrens Hospital, CIC 359, A Tuncer, D Ucak (24 (20) 24/0)
Ankara, University School of Medicine Ibi Sina Hospital (hem), CIC 617, G Girman, M Aral (114 (123) 63/51)
Ankara, University o Ankara (pedos), CIC 620, E Ural (20 (20) 15/3)
Ankara, Numune Education and Research Hospital, CIC 691, M Yalily (42 (42) 19/23)
Ankalya, Akdeniz University Hospital (pedos), CIC 618, MA Yesilpok, V Hizlar, A Kupci (37 (39) 34/3)
Antalya, Akdeniz University Hospital (hem), CIC 685, L Uzdar (27 (32) 13/4)
Aydin, Adnan Menderes University Medical Faculty (hem), CIC 187, Z Bolkman (6 (4) 0/4)
Bakali (Adana), Cukurova University Hospital (ads, onco), CIC 821, A Tanyeli (6 (8) 6/0)
Bakali (Adana), Cukurova University Hospital (ads, onco), CIC 821, B Sahin (16 (16) 0/16)
Borneo-Izmir, Ege University Medical Faculty (pedos), CIC 621, S Kanoy (17 (17) 0/7)
Borneo-Izmir, Ege University Medical Faculty (ads, onco), CIC 628, S Cagirgan (66 (67) 15/51)
Estekler, Osmangazi University, CIC 666, Z Gubas (32 (32) 12/20)
Istanbul, Marmara University (hem, onco), Altunizade, CIC 714, T Akdoglu (no report)
Istanbul, University of Istanbul, CIC 760, S Kalayoglu-Besikci (34 (35) 18/16)
Istanbul, Cerrahpasa Medical School, CIC 761, B Fehranoglu, T Soyalt, M Gem Cor (42 (42) 13/29)
Istanbul, University of Istanbul Pediatric BMT Unit (pedos, hem, onco), CIC 400, S Arak, O Gulyar (12 (12) 9/3)
Istanbul, GATA Haydarpasa Egitim Hasti (hem, onco), CIC 687, A Ozalak (0 (0) 0/0)
Istanbul, Yildiztepe University Hospital (hem, onco), CIC 919, Y Koc (35 (37) 24/15)
Izmir, Dokuz Eylul University (onco), CIC 688, H Ozcan, U Yilmaz (18 (18) 1/17)
Kayseri, Erciyes University Hospital (onco, hem), CIC 627, A Ural, M Celik (56 (56) 34/22)
Trabzon, Karadoc Technical University, (hem), CIC 170, E Ovadi (19 (19) 5/16)

Ukraine: (two teams: 27 (31) 0/27)
Kiev, Kiev City BMT Center, CIC 176, E Karzamenech, V Khomenko, I Korshenkov, B Borodkin (27 (31) 0/27)
Kiev, Kiev Regional Oncologic Hospital (pedos, hem, onco), CIC 177, S Donska, O Ryzhak (no report)

United Kingdom: (52 teams: 2447 (2588) 1016/1431)
Aberdeen, The Royal Infirmary (hem), CIC 344, DJ Cutliff (16 (16) 5/11)
Bangor, Gwynedd Hospital (hem, onco), CIC 736, D Edwards (16 (17) 0/16)
Bath, Royal United Hospital (hem), CIC 619, C Knoblauch (14 (14) 0/14)
Belfast, Belfast City Hospital (hem, onco), CIC 258, F Jones, TCM Morns, P Abram (43 (46) 7/36)
Birmingham, Heartlands Hospital (hem), CIC 284, RW Milligan (33 (37) 12/21)
Birmingham, Queen Elizabeth Hospital (hem), CIC 387, C Craddock, P Mahendra (143 (140) 70/73)
Birmingham, The Birmingham Childrens Hospital (hem), CIC 781, PJ Darbyshire (42 (43) 31/11)
Bournemouth, Royal Bournemouth Hospital (hem), Poole Hospital, Dorset Cancer Centre and Salisbury District Hospital, CIC 765, S Killick, J Curts (23 (26) 0/23)
Bristol, Royal Hospital for Children (allo, adv, ped), CIC 386:1, JM Cormish, D Marks (66 (67) 64/2)
Bristol, Avon Haematology Unit (auto), CIC 386:2, R Evely, J Bird (24 (27) 0/24)
Cambridge, Addenbrooke's Hospital (hem), CIC 556, C Crawley, RE Marcus, J Cragg, H Balston, T Chapman (76 (78) 28/50)
Cardiff, University Hospital of Wales (hem), CIC 303, KMO Wilson, AK Burnett, JA Whitaker, CH Poynton (68 (72) 31/57)
Cheltenham, Cheltenham General Hospital, CIC 398, E Blundell (20 (20) 0/20)
Coventry, University Hospital & Warwickshire NHS Trust, J Mills (17 (17) 0/17)
Dudley, The Dudley Group of Hospitals NHS Trust (hem), CIC 405, S Fernandez (3 (3) 0/3)
Dundee, Ninewells Hospital (hem), CIC 719, D Meklejohn (7 (7) 0/7)
Edinburgh, Western General Hospital, (hem) CIC 228, PRE Johnson, J Davies, F Scott, PH Ruddle, P Shepherd (37 (37) 10/27)
Exeter, Royal Devon and Exeter Hospital (hem), CIC 571, C Rudin (12 (13) 9/12)
Glasgow, Royal Infirmary, CIC 244, IG McQuaker, A Parker (75 (80) 40/39)
Glasgow, The Western Infirmary (hem), CIC 325, T Fitzsimons (25 (25) 0/25)
Glasgow, Royal Hospital for Sick Children (hem), CIC 707, B Gibson (17 (19) 12/3)
Leeds, St James's University Hospital, The General Infirmary, Pinderfields Hospital CIC 254, G Cook, S Kinsey, MC Galvin (113 (182) 32/81)
Leicester, Royal Infirmary (hem), CIC 713, AE Hunter (64 (65) 18/46)
Liverpool, Royal Liverpool University Hospital (hem), CIC 501, RE Clark, A Pettitt (52 (53) 16/36)
Liverpool, Alder Hey, CIC 773, M Caswell (11 (11) 7/4)
London, Hammersmith Hospitals NHS Trust, CIC 203, J Apperley, E Olavanna, E Kafer, A Rahentulla, R Seydlo (105 (122) 30/75)
London, Royal Free Hospital (hem), CIC 216, S Mackinnon (69 (70) 43/26)
London, Royal Marsden Hospital (hem), CIC 218, M Potter (149 (160) 58/91)
London, University College Hospital (hem), CIC 224, K Thomson (131 (134) 51/90)
London, Great Ormond Street Hospital, CIC 243, P Veys (54 (64) 42/12)
London, The London Clinic (hem), CIC 263, M Potter, P Gravett (8 (10) 2/6)
London, St George's Hospital (hem), CIC 539, J Marsh, S Ball, EC Gordon-Smith (24 (20) 14/10)
London, Guy's Hospital (hem), CIC 721, M Kazmi (44 (45) 15/29)
London, King's College (hem), CIC 763, A Pagliuca (108 (116) 72/36)
London, St Bartholomew's, CIC 768 and the Royal London Hospital, J Cribben, J Cavenagh, S Agrawal, T Lister (82 (92) 29/53)
London, St Mary's Hospital, CIC 866, J de La Fuente (11 (11) 11/0)
Manchester, Royal Children's Hospital, CIC 521, R Wyn (32 (33) 29/3)
Manchester, The Royal Infirmary, CIC 601, JA Yin (37 (39) 29/8)
Manchester, Christie Hospital (hem), CIC 780, E Liakopoulos (65 (76) 24 (41)
Newcastle upon Tyne, Royal Victoria Infirmary and the Sunderland Royal Hospital, CIC 276, GJ Jackson, SJ Proctor, P Taylor, A Cant, R Skinner PJ Carey (86 (89) 41/45)
Norwich, Norfolk and Norwich Hospital (hem), CIC 391, M Lawes, G Turner (10 (10) 0/10)
Nottingham, City Hospital, CIC 717, N Russell, JL Byrne, AF Haynes, A McMillan (119 (122) 51/68)
Oxford, John Radcliffe Hospital (hem, onco), Headington and Wycombe General, CIC 255, TJ Littlewood, C Burch, C Mitchell, C Harston, GH Hall, J Wainwright (72 (74) 30/42)
Plymouth, Derriford Hospital, CIC 823, MD Hamon (45 (47) 13/22)
Salford, Hope Hospital, JB Houghton (1 (3) 0/3)
Sheffield, Royal Hallamshire Hospital—J Snowden; Weston Park Hospital—L Evans; Rotherham General Hospital—H Barker and the Children's Hospital—A Vora, CIC 778:1/23 (63 (68) 27/36)
Somerset, Taunton and Somerset Hospital, S Bokam, SA Johnson (10 (10) 0/10)
Southampton, CRC Wessex, CIC 704, K Orchard, A Duncombe, J Kohler (74 (79) 24/50)
Stoke-on-Trent, University Hospital of North Staffordshire (hem), CIC 394, R Chasty (8 (8) 0/8)
Swansea, Singleton Hospital, Skett, S Al Ismail (9 (9) 9/9)
Swindon, Great Western Hospital (hem), CIC 608, NE Blesing, A Gray, S Green, A Koster (8 (8) 0/8)

Total Europe 2006: 25 059 (28 740) 9661/15 398
November 2007
Rituximab maintenance improves clinical outcome of relapsed/resistant follicular non-Hodgkin lymphoma in patients both with and without rituximab during induction: results of a prospective randomized phase 3 intergroup trial


We evaluated the role of rituximab (R) both in remission induction and maintenance treatment of relapsed/resistant follicular lymphoma (FL). A total of 485 patients were randomized to induction with 6 cycles of cyclophosphamide, doxorubicin, vinristine, and prednisone (CHOP) (every 3 weeks) or R-CHOP (R: 375 mg/m² intravenously, day 1). Those in complete remission (CR) or partial remission (PR) were randomized to maintenance with R (375 mg/m² intravenously once every 3 months for a maximum of 2 years) or observation. R-CHOP induction yielded an increased overall response rate (CHOP, 72.3%; R-CHOP, 85.1%; \( P < .001 \)) and CR rate (CHOP, 15.8%; R-CHOP, 29.6%; \( P < .001 \)). Median progression-free survival (PFS) from first randomization was 20.2 months after CHOP versus 33.1 months after R-CHOP (hazard ratio [HR], 0.65; \( P < .001 \)). Rituximab maintenance yielded a median PFS from second randomization of 51.5 months versus 14.9 months with observation (HR, 0.40; \( P < .001 \)). Improved PFS was found both after induction with CHOP (HR, 0.33; \( P < .001 \)) and R-CHOP (HR, 0.54; \( P = .004 \)). R maintenance also improved overall survival from second randomization: 85% at 3 years versus 77% with observation (HR, 0.32; \( P = .011 \)). This is the first trial showing that in relapsed/resistant FL rituximab maintenance considerably improves PFS not only after CHOP but also after R-CHOP induction.

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Introduction

For patients with follicular lymphoma (FL) chemotherapy alone has not resulted in improved overall survival (OS) over the past 30 years. Although in most patients complete remissions (CRs) or partial remissions (PRs) can be obtained with either single agents or combination chemotherapy, the clinical course is characterized by a high relapse rate. After relapse, both the response rate and relapse-free survival after subsequent salvage treatment regimens steadily decrease, resulting in a median survival of only 4 to 5 years after first relapse. Therefore, new treatment modalities resulting in increased progression-free survival (PFS) and OS are urgently required. Optimal treatment of patients relapsed after 1 or 2 chemotherapy regimens is largely unknown.

Rituximab (R) is a chimeric murine/human anti-CD20 monoclonal antibody capable of killing CD20+ lymphoma cells. Effector mechanisms include complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity, and possibly direct induction of apoptosis. In the nonrandomized pivotal study in 166 relapsed low-grade lymphoma patients, monotherapy with rituximab resulted in a response rate of 48%, with a 62% complete remission (CR) rate and a median time to progression in responding patients of 13 months. Toxicity was generally mild to moderate (grade 1 or 2) and occurred primarily with the first infusion. In a subsequent phase 2 study, the combination of R with cyclophosphamide, doxorubicin, vinristine, and prednisone (R-CHOP) was shown to be safe and effective.

Treatment results in FL might not only be improved by more effective induction regimens, but also by maintenance treatment defined as continued treatment beyond induction therapy. Maintenance treatment with cytotoxic agents has been shown to improve PFS but not OS. This prolongation of PFS was achieved at the cost of increased toxicity, reduced patient well-being, and increased risk of secondary malignancies. In a recent meta-analysis, interferon maintenance treatment showed a survival benefit in FL when given in conjunction with intensive chemotherapy and at certain dose levels. However, the benefit of maintenance was not consistent across all studies, and toxicity was considerable.

From the European Organisation for Research and Treatment of Cancer (EORTC) Lymphoma Group, Hemato-Oncologie voor Volwassenen Nederland (HOVON), National Cancer Institute of Canada (NCIC) Clinical Trials Group (CTG) (Canada), British National Lymphoma Investigation (BNLI), Australasian Leukaemia and Lymphoma Group (ALLG), Nordic Lymphoma Group (NELG), and EORTC Data Center.


A complete list of the members of the Intergroup Collaborative Study (EORTC-20891) appears in "Appendix."

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The authors declare no competing financial interests.

M.H.J.v.O., R.K., R.E.M., W.W., E.K., and A.H. formed the writing committee designing the study and were principal investigators of the participating lymphoma groups; R.D.G. and A.J. performed the major part of the pathology review; M.K.H.V., A.V., and H.H. included the largest number of patients calculated per center; M.G.V. performed all statistical analyses; I.T. and C.F. performed all data analysis; and M.H.J.v.O. wrote the paper.

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Because of its efficacy as monotherapy and its favorable pharmacokinetic and safety profile, maintenance treatment with rituximab might be both effective and well-tolerated.

In view of (1) the efficacy of rituximab monotherapy in relapsed low-grade lymphoma,11 (2) the feasibility of combining rituximab with cytotoxic drugs,12 and (3) the theoretical potential of such combinations to clear minimal residual disease, we decided in 1998 to launch a phase 3 randomized clinical trial in patients with relapsed or resistant FL with 2 main objectives: first, to compare response rates with CHOP and R-CHOP and, second, to establish the effect of maintenance treatment with rituximab on progression-free survival (PFS).

Patients, materials, and methods

Patients

This randomized (1:1) open-label phase 3 intergroup study was conducted at 130 centers in Canada, Australia/New Zealand, Europe, and South Africa from November 1998 to April 2004. Patients eligible for the study were older than 18 years of age with a CD20+ grade 1 to 3 FL, Ann Arbor stage III or IV at initial diagnosis, and relapse after or resistance to a maximum of 2 monotherapies containing systemic chemotherapy regimens. A previous regimen was defined as at least 2 months of single-agent therapy (eg, chlorambucil) and at least 2 consecutive courses of polychemotherapy (eg, cyclophosphamide, vincristine, and prednisolone [CVP] or purine analogs). Patients had to have at least 1 bidimensional measurable mass by either clinical or radiologic examination. World Health Organization (WHO) performance status had to be 2 or below. Major exclusion criteria were prior treatment with chemotherapy, rituximab, or autologous or allogeneic stem cell transplantation; more than 10 × 10^9/L circulating tumor cells; histologic transformation; known HIV positivity; symptomatic central nervous system (CNS) lymphoma; IgG levels below 3 g/L; and severe concomitant disease. Patient information and written informed consent were obtained according to the rules of the respective country and institute. The study was conducted according to the Declaration of Helsinki and the Guidelines for Good Clinical Practice.

Study design and treatment

Eligible patients were randomized to remission induction with either 6 cycles of standard CHOP (cyclophosphamide 750 mg/m^2 intravenously, day 1; doxorubicin 50 mg/m^2 intravenously, day 1; vincristine 1.4 mg/m^2 intravenously, maximum 2 mg, day 1; and prednisone 100 mg orally daily, days 1 to 5; cycle every 3 weeks) or CHOP plus rituximab (R) (375 mg/m^2 intravenously at day 1 of each cycle of CHOP). After 2 cycles of CHOP with or without R, patients were evaluated for response. Those with stable disease or progression went off study. Responders received another 3 cycles of the assigned treatment. Patients with a CR or a PR after 6 cycles of therapy underwent a second randomization to either no further treatment [observation] or maintenance treatment with rituximab (375 mg/m^2 intravenously once every 3 months until relapse or for a maximum of 2 years). Exclusion criteria for second randomization were no CR or PR upon induction treatment, IgG levels below 3 g/L, and active infection.

First randomization was stratified by center, previous treatment with purine analogs, age, number of previous induction treatments, and best response previously obtained (CR/PR/no change [NC]/progressive disease [PD]), time since diagnosis (more than 2 years or 2 years or less), and bulky disease (more than 10 cm or 10 cm or less) using a minimization procedure.

The second randomization was stratified according to the treatment allocated by the first randomization, the quality of the response obtained after induction (CR/PR), and center. Responses after induction treatment were evaluated by physical examination, hematology and chemistry, computed tomography (CT) scans (obligatory), and bone marrow biopsies (when indicated) and assessed according to the Lymphoma Expert's Confirmation of Response (LEXCOR) criteria.18 Patients lacking bone marrow evaluation but with evidence of disease on physical examination and CT scans were scored as partial remissions.

During the 2 years of R maintenance/observation, physical examination and hematology and chemistry were performed at least every 3 months and thereafter once every 4 to 6 months. In this large multicenter international study it was decided to adhere as much as possible to the daily practice in the participating countries. Thus, during maintenance/observation CT and bone marrow examinations were performed only on indication. A central pathology review was performed by all participating groups.

The trial was designed to detect a 10% difference (from 90% to 80%) in the overall response rate to induction chemotherapy and to recruit 500 patients (alpha = 0.05, beta = 0.22, 2-sided test). The final analysis of maintenance was performed after 501 progressions or deaths to detect a 14% difference in the 2-year PFS (from 40% to 54%; alpha = 0.05, beta = 0.2, 2-sided test). An interim analysis of safety was planned after inclusion of 50 patients and 2 interim analyses of efficacy after inclusion of 300 and 400 patients (Haybittle and Peto strategy, P < .001).

Statistical analysis

All primary analyses were conducted following the intention to treat (ITT) principle. The primary end point for the induction phase was the response to treatment. Secondary end points were PFS and OS from first randomization. Response rates were compared using the Mantel-Haenszel test for trend on 4 ordered categories (CR/PR/NC/ND). For the primary analysis, nonassessable patients were excluded. For sensitivity analyses, nonassessable cases were considered as progressions. The primary end point for the maintenance phase was PFS defined as interval between the date of second randomization and date of first relapse, progression, or death, and the secondary end point was OS from second randomization. The principal analysis of PFS and OS was done with the log-rank test and sensitivity analyses done with Cox regression analysis with adjustment for type of induction treatment and response. Kaplan-Meier curves were calculated to graphically show the differences between the treatment arms. All P values given are 2-sided.

In February 2004, a preplanned second interim analysis of the present study was reviewed by the Independent Data Monitoring Committee (IDMC) of this study. At that time, 461 patients had been included (569 evaluable for response) and 319 patients had been randomized for maintenance treatment (268 evaluable). The results revealed that the primary end points for both the induction and maintenance phase of the study had been reached, and the formal criteria for stopping the trial had been met. Subgroup analysis as requested by the IDMC continued the benefit of R maintenance in the CHOP subgroup but not yet in the R-CHOP subgroup. It was therefore suggested to amend the protocol with all patients receiving R-CHOP for induction treatment followed by randomization to R maintenance therapy or no further treatment. Hence, recruitment to the trial was suspended in April 2004 during preparation of a major protocol amendment. In the meantime, all data were retrospectively monitored on site, and all pending queries were solved to perform a final analysis including all patients recruited to the study by April 2004. Because the study was conducted at 130 sites and by 8 clinical study groups, the monitoring and data cleaning process was only completed in September 2005. Thus, an updated data set with additional 19 months of median follow-up was available for the final analysis. After reviewing the data of this final analysis, the IDMC recommended not to reopen the trial because the primary question of the amended protocol had already been answered.

Results

Patients

A total of 474 patients with relapsed/resistant FL were randomly assigned to receive induction treatment with CHOP or R-CHOP. Nine patients had to be excluded because of missing informed consent forms. Therefore, all analyses are restricted to 465 patients.
Table 1. Baseline characteristics according to treatment group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CHOP</th>
<th>R-CHOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>231</td>
<td>254</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>55 (27-79)</td>
<td>54 (26-60)</td>
</tr>
<tr>
<td>Male/female, %</td>
<td>51/48</td>
<td>42/54</td>
</tr>
<tr>
<td>Stage IV at diagnosis, %</td>
<td>67</td>
<td>66</td>
</tr>
<tr>
<td>Bulky disease, more than 10 cm, %</td>
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<td>15</td>
</tr>
<tr>
<td>B symptoms, %</td>
<td>27</td>
<td>26</td>
</tr>
<tr>
<td>FLIPI score,*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>3 or higher</td>
<td>37</td>
<td>37</td>
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<tr>
<td>Time from initial diagnosis, %</td>
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<tr>
<td>2 y or less</td>
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<td>45</td>
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<tr>
<td>More than 2 y</td>
<td>52</td>
<td>50</td>
</tr>
<tr>
<td>Prior treatment, %</td>
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<td></td>
</tr>
<tr>
<td>Single, best</td>
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<td>39</td>
</tr>
<tr>
<td>Polychemotherapy</td>
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<td>35</td>
</tr>
<tr>
<td>2 regimens</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Best response to prior treatment, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>31</td>
<td>33</td>
</tr>
<tr>
<td>PR</td>
<td>58</td>
<td>51</td>
</tr>
<tr>
<td>Refractory</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>SD</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PD</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

SD indicates stable disease.

* Assessed retrospectively.

(231 CHOP and 234 R-CHOP). Recruitment was stopped after the preplanned second interim analysis for efficacy because the criteria for early discontinuation were met both for induction and maintenance. Baseline demographics and other characteristics of the 2 groups were well balanced (Table 1). Because the Follicular Lymphoma International Prognostic Index (FLIPI) was only published in 2004,18 the FLIPI score was assessed retrospectively for our patients and thus was not used as a stratification factor. However, both study arms were well balanced, with 70% of the patients having intermediate (FLIPI score 2) or high-risk (FLIPI score 3 or higher) disease at study entry (Table 1). According to local pathology, 98% of the patients had FL. Central pathology data are available for 82% of all patients. The overall concordance rate between local and central assessment for all subtypes of FL was 93% in both treatment arms.

In both groups, about 80% had received only one prior treatment, almost equally consisting of single-agent therapy or polychemotherapy. In both arms, only 9% of the patients had been treated previously with purine analogs. Best response to prior treatment was similar in both study arms. A total of 17% and 16% of patients were resistant to their prior treatment in the CHOP and R-CHOP arms, respectively (Table 1). Three randomized patients never started protocol treatment—1 because of rapid progression and 2 refusals. The 6 cycles of protocol therapy could be completed in 81% of the patients in the CHOP arm and in 85% in the R-CHOP arm. Dose density for doxorubicin and cyclophosphamide was similar in both arms. Most protocol discontinuations occurred at the time of the first response evaluation, after the third cycle of treatment.

The 334 patients randomized to the maintenance phase were well balanced for baseline characteristics at study entry, FLIPI score (2 or higher in 70% and 66% in the observation and maintenance arms, respectively), type of induction treatment received, and response to induction (in both arms, 39% CR and 71% PR). In both arms, more patients had received R-CHOP during induction (59% in the observation arm and 55% in the maintenance arm), reflecting the higher efficacy of R-CHOP as compared with CHOP in terms of response induction. Maintenance treatment was started a median of 7 weeks (range, 3 to 16 weeks) after the end of the last induction cycle.

Efficacy: induction phase

The addition of rituximab significantly increased both overall response and complete remission rates. Overall response rates were 72.3% and 85.1% after CHOP and R-CHOP induction treatment, respectively (P < .001). The CR rate was 15.6% in patients receiving CHOP and 29.5% in patients treated with R-CHOP (P < .001) (Table 2). The partial response rate was 56.7% in the CHOP arm and 55.6% in the R-CHOP arm (non-significant [NS]). With a median follow-up from first randomization of 39.4 months, median PFS from first randomization was 20.2 months in the CHOP group versus 33.1 months in the R-CHOP group (P < .001, log-rank test; Figure 1A). Hazard ratio (HR) for the R-CHOP group was 0.65. OS at 5 years from first randomization was 71.9% in the CHOP arm and 82.5% in the R-CHOP arm (P = .096, log-rank test; HR, 0.74; Figure 1B).

Efficacy: maintenance phase

Of the 366 patients having responded to induction treatment (with either CHOP or R-CHOP) 32 were not randomized for maintenance treatment—17 because of low IgG levels (9 in the CHOP arm, 8 in R-CHOP); 8 patients because they were still on CHOP induction when the trial was put on hold because of the results of the first interim analysis (these patients received R maintenance treatment on a compassionate use basis; they were included in the analysis of response to induction but were excluded from the analysis of maintenance treatment); 1 because of a secondary neoplasia; and 1 because of active infection. There were 2 refusals and 2 ineligibilities due to administrative problems.

A total of 334 eligible patients were randomly assigned to R maintenance treatment (n = 167) for 2 years or observation (n = 167). In each study arm, 1 patient did not start allocated treatment because of progression immediately after randomization. At the time of last follow-up, 41 patients were still under maintenance treatment or observation. With a median follow-up from second randomization of 33.3 months, median PFS from second randomization was 51.5 months in the R maintenance arm versus 14.9 months in the observation arm (P < .001, log-rank test). The hazard ratio for R maintenance treatment compared with observation was 0.40; P < .001 (Figure 2A). Because the difference in PFS was highly significant, a further analysis was carried out to evaluate whether the benefits of maintenance applied to patients treated both with CHOP and R-CHOP. After CHOP induction, R maintenance resulted in a median PFS from second

Table 2. Response to induction treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CHOP</th>
<th>R-CHOP</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>231</td>
<td>254</td>
</tr>
<tr>
<td>ORR,*%</td>
<td>72.3</td>
<td>85.1</td>
</tr>
<tr>
<td>CR, %</td>
<td>15.6</td>
<td>28.9</td>
</tr>
<tr>
<td>PR, %</td>
<td>56.7</td>
<td>55.6</td>
</tr>
<tr>
<td>SD, %</td>
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<td>5.0</td>
</tr>
<tr>
<td>PO, %</td>
<td>9.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Death, %</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Nonassessable</td>
<td>6.8</td>
<td>6.4</td>
</tr>
</tbody>
</table>

ORR indicates overall response rate.

* P < .001 by the Mantel-Haenszel test for trend.
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A

Overall Logrank test: P < .001

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<tr>
<td>O</td>
<td>149</td>
<td>231</td>
<td>80</td>
<td>42</td>
<td>16</td>
<td>4</td>
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<tr>
<td>N</td>
<td>122</td>
<td>234</td>
<td>198</td>
<td>109</td>
<td>69</td>
<td>24</td>
</tr>
<tr>
<td>Number of patients at risk</td>
<td></td>
<td></td>
<td></td>
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</table>

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<tr>
<th>Treatment</th>
<th>CHOP</th>
<th>R-CHOP</th>
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</table>

Figure 1. Effect of addition of R (rituximab) to CHOP remission induction on progression-free survival and overall survival. Kaplan-Meier plots of progression-free survival and overall survival from first randomization. (A) Progression-free survival after CHOP (n = 231) and R-CHOP (n = 234) remission induction treatment. (B) Overall survival after CHOP (n = 231) and R-CHOP (n = 234) remission induction treatment.

randomization of 42.2 months versus 11.6 months in the observation arm (HR, 0.30; P < .001). After R-CHOP induction, the figures were 51.8 months and 23.0 months, respectively (HR, 0.54; P = .004) (Figure 3). Similarly, R maintenance resulted in a highly significant increase in PFS both in patients who had a FR after induction treatment and those who had obtained a CR (data not shown).

R maintenance treatment increased 3-year overall survival rates (from second randomization) from 77.1% in the observation group to 85.1% in the R maintenance group (P = .011, log-rank test; Figure 2B). The hazard ratio for R maintenance compared with observation is 0.52. All sensitivity analyses confirmed the results of the principal analyses.

Safety

Induction. Grade 3-4 neutropenia was the most frequent adverse event (AE); 48.2% grade 3-4 in the CHOP arm and 54.7% in the R-CHOP arm (NS). More patients on R-CHOP experienced grade 3-4 allergy (CHOP, 0 patients; R-CHOP, 8) and skin reactions (CHOP, 17 patients; R-CHOP, 31). Six patients in the CHOP arm and 8 patients in the R-CHOP arm withdrew from treatment because of toxicity. Treatment-related mortality occurred in 2 patients in the CHOP group (1 sepsis, 1 respiratory distress syndrome) and in 1 patient in the R-CHOP group (pneumonia). During induction, hypogammaglobulinemia developed in about 5% of the patients. Indeed, 17 of the 366 responders to induction treatment were not eligible for second randomization because of IgG levels below the predefined threshold of 3 g/L. However, we did not find a correlation between the incidence of bacterial infections and decreased IgG levels.

Maintenance. During R maintenance treatment, neutropenia was the only significant AE: 10.8% in the R maintenance arm versus 5.4% in the observation arm (NS; 95% CI = 0.07, 0.10). This probably contributed to the increased grade 3-4 infection rate: 9% in the maintenance group and 2.4% (P = .009, 95% CI) during observation, with most of these in the ear-nose-throat area. During maintenance 6 patients with therapy-related grade 3-4 adverse events (infection) were hospitalized. They fully recovered.

Only 6 of the 167 patients withdrew from R maintenance treatment because of toxicity (4 of the 6 due to infections). According to protocol, IgG (but not IgA and IgM) levels were measured every 3 months during maintenance treatment/observation. At second randomization the median IgG levels were just below the normal range in both arms (6.6 g/L in the observation arm and 6.5 g/L in the maintenance arm). Whereas during 2 years of observation the median IgG levels increased to within the normal range (7.3 g/L). IgG levels remained stable in the maintenance arm (6.3 g/L). Maintenance dose was delayed in only 1 patient and omitted in 2 patients at least once, because of low (below 3 g/L) IgG levels. No patient had to be withdrawn from R
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RITUXIMAB MAINTENANCE IN FOLLICULAR LYMPHOMA 3299

[Graphs and charts]

Figure 3. Effect of R (rituximab) maintenance treatment on progression-free survival after remission induction with either CHOP or R-CHOP. Kaplan-Meier plots of progression-free survival from second randomization. (A) Progression-free survival after CHOP remission induction (n = 145). (B) Progression-free survival after R-CHOP remission induction (n = 189).

maintenance treatment due to persisting IgG levels below 3 g/L. There were no deaths related to R maintenance treatment.

Discussion

The final analysis of the European Organisation for Research and Treatment of Cancer (EORTC) 20981 Intergroup study has shown several important findings. Firstly, in patients with relapsed/resistant FL, remission induction with R-CHOP results in a highly significant increase in CR rate as compared with CHOP; secondly, R maintenance treatment significantly improves PFS and OS in patients responding to induction treatment; thirdly, R maintenance treatment achieves a considerable increase in PFS not only after remission induction with chemotherapy (CHOP) but also after immunotherapy (R-CHOP).

Since the start of the trial in late 1998, a considerable amount of data on efficacy and safety of rituximab in combination with different chemotherapy regimens as induction therapy for both previously untreated and pretreated patients has been published.2234 There is a strong rationale for this combination because cytotoxic drugs and rituximab both have proven efficacy but different mechanisms of action and nonoverlapping toxicities. In addition, in vitro data have shown that rituximab may increase sensitivity of lymphoma cells to cytotoxic agents.9

In indolent lymphoma, remission induction with the combination of rituximab and chemotherapy has been shown to be superior to chemotherapy alone in several randomized phase 3 trials, both in previously untreated as well as in relapsed patients. In previously untreated patients, the addition of R to chemotherapy results in significantly better overall response and complete remission rates and improved PFS22 23 and OS.22,24 Our finding of a superior CR rate after R-CHOP in relapsed FL patients is in line with the results of the German Low Grade Lymphoma Study Group, which showed in a mixed group of relapsed/refractory indolent non-Hodgkin lymphoma (NHL) and mantle cell lymphoma patients that R-FCM (rituximab-fludarabine, cyclophosphamide, and mitoxantrone) yields significantly higher ORR and CR rates and prolongs PFS and OS when compared with FCM alone.25 In all these studies, addition of rituximab to chemotherapy did not result in increased toxicity.

In the past decades, maintenance therapy over a period of 12 to 24 months after induction treatment was evaluated using cytotoxic agents such as chlorambucil or cyclophosphamide10 11 or interferon-α.12,13,26 However, no consistent long-term benefit in terms of OS could be demonstrated, and prolonged administration of both chemotherapy and interferon-α are associated with significant toxicity and patient inconvenience.

Two randomized trials have investigated the efficacy of induction therapy with single-agent rituximab followed by rituximab maintenance treatment. Hainsworth et al randomized patients with relapsed or refractory indolent NHL to R maintenance or R retreatment at disease progression and found an approximate 4-fold increase in PFS for the former (31.1 months versus 7.4 months).27 However, the rituximab benefit (defined as date of study entry to date of next lymphoma treatment) was similar in both groups (31.3 versus 27.4 months, respectively). Because this was not part of our study, we do not have systematic information on the retreatment of patients who relapsed after either R maintenance or observation. However, because rituximab was registered and available in all participating countries, it has to be assumed that many patients will have received a rituximab-containing regimen, notably those who received rituximab neither during induction nor maintenance.

Indeed, a preliminary analysis showed that in the patients in the observation arm, first post-protocol treatment (n = 85) was R monotherapy in 29% and R-chemo in 19%, versus 11% and 5%, respectively, in patients in the maintenance arm requiring post-protocol treatment (n = 56). The Swiss Group for Clinical Cancer Research (SAKK) 35-98 study showed an almost 2-fold increase in median event-free survival (EFS) by R maintenance in patients with untreated and relapsed FL (from 12 to 23 months).28

The efficacy of R maintenance therapy has also been investigated following treatment with different chemotherapy regimens. In previously untreated patients with indolent lymphoma (Eastern Cooperative Oncology Group [ECOG] 1496). R maintenance treatment after remission induction with CVP increased PFS by almost 3 years and improved OS in patients with high tumor burden.29 In all these studies, R maintenance treatment was well tolerated and did not lead to significantly higher rates of neutropenia, thrombocytopenia, and/or infection as compared with observation.

Our study is the first large randomized trial to show that in patients with relapsed/resistant FL, R maintenance treatment achieves a statistically highly significant and clinically very relevant improvement in PFS after induction treatment with chemotherapy plus rituximab. For the pooled CHOP and R-CHOP patients, R maintenance also improved OS: 85% at 3 years versus 77% with observation (HR, 0.52; P = .011). Of course, follow-up is still rather short for patients with FL, and longer follow-up is required to know whether the survival benefit will stand. Recently, a preliminary report of a randomized study by Hiddemann et al in a mixed population of relapsed FL and MCL also showed a
significant improvement in response duration for patients receiving R maintenance after induction therapy with R-FCM (n = 119). In relapsed FL, there has only been one randomized trial comparing chemotherapy and autologous stem cell transplantation. Although the number of patients was small and not balanced as to prognostic factors between the study arms, a clear benefit for autologous stem cell transplantation as to PFS and OS was shown. In view of the excellent PFS obtained with R-CHOP induction followed by R maintenance, future trials in relapsed FL should compare this (or a comparable) regimen with an optimal transplantation approach: R-chemotherapy induction and R-myeloablative treatment, and R maintenance after transplantation. This probably also applies to future trials in FL of autologous transplantation in first remission.

In conclusion, we have shown that R maintenance treatment results in a major improvement in PFS both after chemotherapy and immunomodulatory treatment, and, most importantly, also in a better OS. Questions still to be answered related to the optimal schedule (e.g., single infusions every 2 to 3 months or weekly infusions every 6 months) and duration of R maintenance (e.g., 6 weeks or until progression), and whether the results of R-chemotherapy induction and R maintenance in relapsed/refractory FL will be similar in patients who have already received prior rituximab-containing regimens.

Acknowledgments

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Appendix

The following investigators (listed in alphabetical order) included patients in the study.

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References

Prognostic Factors in Allogeneic Bone Marrow Transplantation for Multiple Myeloma

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Purpose: To analyze prognostic factors for allogeneic bone marrow transplantation (BMT) in multiple myeloma.

Patients and Methods: One hundred sixty-two reports of allogeneic matched sibling-donor transplants in multiple myeloma received by the European Group for Blood and Marrow Transplantation (EBMT) registry between 1983 and early 1993 were analyzed for prognostic factors. End points were complete remission, survival, and duration of complete remission.

Results: Following BMT, 44% of all patients and 60% of assessable patients entered complete remission. The overall actuarial survival rate was 32% at 4 years and 28% at 7 years. The overall relapse-free survival rate of 72 patients who were in complete remission after BMT was 34% at 6 years. Favorable pretransplant prognostic factors for survival were female sex (41% at 4 years), stage I disease at diagnosis (52% at 4 years), one line of previous treatment (42% at 4 years), and being in complete remission before conditioning (64% at 3 years). The subtype immunoglobulin A (IgA) myeloma and a low $\beta_2$–microglobulin level (< 4 g/L) also tended to have a favorable prognostic impact. The most important posttransplant prognostic factor was to enter a complete remission. Grade III to IV graft-versus-host disease (GVHD) was associated with poor survival.

Conclusion: Patients with a low tumor burden who respond to treatment before BMT and are transplanted after first-line therapy have the best prognosis following BMT.


MULTIPLE MYELOMA is a malignant disorder with a median survival duration of less than 3 years after conventional chemotherapy. However, survival is highly variable. Some patients die within months, while occasional patients survive more than 10 years. This heterogeneity in the prognosis of multiple myeloma has encouraged studies of prognostic variables. A high tumor burden, high C-reactive protein level, high plasma-cell thymidine-labeling index, elevated $\beta_2$–microglobulin level, low serum albumin level, and low platelet count have been associated with extremely poor survival following conventional chemotherapy. Therefore, such variables have been used to select patients for more intensive treatment. However, only a few studies have attempted to delineate prognostic factors of importance for the outcome of patients treated with high-dose chemotherapy followed by either autologous bone marrow transplantation (ABMT) or allogeneic bone marrow transplantation (BMT).

In a previous study by the European Group for Blood and Marrow Transplantation (EBMT) of 90 patients with multiple myeloma who underwent BMT, a number of factors were found to influence long-term survival, including stage I disease at diagnosis, being in complete remission at transplantation, and having received only a few lines of treatment pretransplant. The first patients reported to the EBMT registry in 1983 have now been monitored for up to 10 years. In the present updated report of 162 patients in the EBMT registry, new variables of prognostic importance have been found.

PATIENTS AND METHODS

Patients

One hundred sixty-two patients with multiple myeloma who received a bone marrow graft from an human leukocyte antigen (HLA)-compatible sibling donor between 1983 and early 1993 were reported to the EBMT registry; 92 were men and 70 were women. The median age was 43 years (range, 23 to 59). The myeloma subtype was immunoglobulin G (IgG) in 80 patients, IgA in 33, light chain in 31, and IgD in two. Five patients had nonsymptomatic multiple myeloma, and three had plasma-cell leukemia. The reports were incomplete in eight patients.

At diagnosis, 22 patients were in stage I, 30 in stage II (one IIB), and 109 in stage III (23 IIB); stage was not reported in one patient. The median time from diagnosis to BMT was 14 months (range, 3 to 166).

Treatment Before BMT

Treatment before BMT varied according to center. Forty-one patients received conventional therapy with intermittent melphalan plus prednisolone as first-line treatment and 121 patients received other drug combinations, most of which contained melphalan or cyclo-
phosphamidase in addition to other drugs. Thirty-six different combinations of drugs were used for second-line treatment and 21 combinations were used for subsequent treatment modalities. Twenty-six patients entered complete remission (defined as the absence of detectable monoclonal Ig in serum and of detectable free light chains in urine on either conventional immunoelectrophoresis or immunofixation, as well as the absence of apparent myeloma cells in the marrow on conventional cytologic analysis) after first-line treatment. Sixty-seven patients had a partial remission (defined as a decrease of serum Ig levels to a concentration less than 50% of pretreatment value, decrease in urine light-chain excretion to < 0.2 g/24 h, or both, combined with a hemoglobin value > 90 g/L, serum albumin level > 30 g/L, and serum calcium level < 2.61 mmol/L). Thirty-eight patients had stationary disease that did not respond to treatment, and in 20 patients the disease progressed (data were lacking for 11 patients). Ninety-six of these patients were later given second-line treatment, and of these, 46 patients received third-line treatment because of poor response or progressive disease. Thus, at the time of conditioning for BMT, 18 patients were in complete remission, 56 were in partial remission, 22 had stable disease that did not respond to further treatment, 14 had primary refractory disease, 25 had progressive disease despite treatment, and 14 were in relapse following complete or partial remission. Data were incomplete in three patients.

**Conditioning Treatment for BMT**

The conditioning for BMT was total-body irradiation (TBI) plus cyclophosphamide alone in 55 patients, TBI plus cyclophosphamide and melphalan in 27 patients, and TBI plus other drug combinations in 35 patients. Forty-five patients received high-dose chemotherapy without TBI. Twenty-five of these patients were conditioned with busulfan plus cyclophosphamide.

**Prevention of Graft-Versus-Host Disease**

Treatment for the prevention of graft-versus-host disease (GVHD) varied. Twenty-one different combinations of T-cell depletion, methotrexate, cyclosporine, prednisone, prednisolone, and cyclophosphamide were used. The single most common combination was cyclosporine plus methotrexate, which was used in 74 patients. Other drug combinations without T-cell depletion were used in 3 patients. T-cell depletion combined with drugs was used in 41 patients and alone in 13 patients. In one patient, information was lacking. Evaluation of GVHD was possible in 134 patients, of whom 46 received T-cell-depleted marrow, alone or in combination with drugs.

**Statistical Analysis**

Comparison of frequencies of complete remission was made with the conventional χ² test or, when small numbers made the χ² test improper, with Fisher's exact test. Survival curves were generated according to the Kaplan-Meier method and were tested with the log-rank test. Kaplan-Meier curves were dichotomized when less than five patients were under observation. Multivariate survival analyses were made with proportional hazards regression, with all quantitative variables dichotomized into qualitative variables.

**RESULTS**

**Response to BMT**

Remission status posttransplant was assessable in 121 patients. Forty-one patients either died before engraftment or were not yet assessable for engraftment. Of 121 patients who could be evaluated, 72 were in complete remission following BMT, of whom 16 were already in complete remission before conditioning for BMT. The median time from BMT to complete remission was 3 months, and in 90% of patients who entered complete remission, the monoclonal component had disappeared at 12 months posttransplant. However, in a small number of patients, the monoclonal Ig persisted for longer time periods, and in two patients it persisted for 36 months then disappeared.

The response to BMT was highly dependent on factors before conditioning. Forty-nine of 64 patients who were on first-line treatment at the time of BMT were assessable for complete remission, and 36 of these 49 patients entered a complete remission, while only 19 of 41 evaluated patients who underwent transplantation while receiving second-line treatment and 16 of 29 evaluated patients who underwent transplantation while receiving third-line or later treatment entered a complete remission (Table 1). The difference in complete remission rate between those who were on first-line treatment compared with those who were on second or later lines of treatment was highly significant ($P = .008$).

The status immediately before conditioning was not significantly predictive for response to BMT, except for patients who were in complete remission before transplantation (Table 2). For those who were not in complete remission immediately before conditioning, 24%, 36%, and 50%, respectively, of the patients who had progressive disease, primary refractory disease, or stable but nonresponsive disease entered a complete remission following BMT.

Other factors that were important predictors of response included stage of disease at diagnosis and patient sex. Irrespective of whether patients were transplanted in later stages of disease, those who were diagnosed in stage I had a significantly higher response rate than those who expressed no evidence of disease.

<table>
<thead>
<tr>
<th>No. of Treatment Lines Pre-BMT</th>
<th>Total No. of Patients</th>
<th>No. of Evaluated Patients</th>
<th>CR Following BMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>49</td>
<td>36 56 73</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>41</td>
<td>19 38 46</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>29</td>
<td>16 35 55</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>121</td>
<td>72 44 60</td>
</tr>
</tbody>
</table>

Abbreviation: CR, complete remission.
were diagnosed in stage II or stage III ($P < .05$) (Table 3). IgG myeloma had a significantly poorer complete remission rate than other subtypes (Table 4). Male patients also had a poorer response than females.

**Bone Lesions**

Bone lesions could be evaluated in 98 patients by roentgenography. Patients who did not survive day 100 were not assessable. Twenty-two of 98 patients had a normal roentgenographic picture before transplantation; 17 did not change following transplantation, while five progressed. Twenty-three patients had minor lytic lesions or osteoporosis; six improved, 10 were stationary, and seven progressed. Fifty-three patients had major lytic lesions; six improved and 47 were stationary. Thus, in 57 of 76 patients who had bone changes before BMT, the roentgenographic bone pattern did not change following BMT.

**GVHD**

One hundred thirty-four patients could be evaluated for GVHD. It was absent in 50 (37%). Forty-three had grade I, 29 grade II, six grade III, and six grade IV. Because of the many various GVHD prevention methods used, it was difficult to analyze any specific preventive method separately. However, if T-cell depletion was included in the preventive method, alone or together with other treatment modalities, the fraction of patients who had grade II to IV GVHD was significantly less than if no T-cell depletion was used ($P = .02$) (Table 5).

**Survival and Disease-Free Survival**

The overall median survival duration after BMT was 17 months (Fig 1). The 4-year survival rate was 32% and the 7-year survival rate 28%. One patient has survived for 10 years.

**Univariate Analysis**

Females had significantly better survival than males ($P = .04$) (Fig 2), but there was no significant difference in survival between patients less than 40 years of age and those $\geq 40$ years ($n = 55$) ($P = .18$).

Patients with stage I disease at diagnosis had a significantly better survival than those diagnosed in stages II and III ($P = .05$) (Fig 3). Patients with IgA myeloma tended to have better survival than patients with IgG myeloma ($P = .08$) and those with light-chain myeloma ($P = .28$) (Fig 4). There was no apparent difference between patients with $\lambda$- or $\kappa$-chain myeloma.

The level of $\beta_2$-microglobulin was measured in 45 patients at the time of diagnosis. There was a tendency for better survival in patients who had $\beta_2$-microglobulin values less than 4 mg/L as compared with those who had a higher value (Fig 5).

Patients who had received only one line of treatment had significantly better survival than those who had re-

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**Table 2. Complete Remission by Status at Conditioning**

<table>
<thead>
<tr>
<th>Status at Conditioning Before BMT</th>
<th>Total No. of Patients</th>
<th>No. of Evaluated Patients</th>
<th>CR Following BMT</th>
<th>% of Total</th>
<th>% of Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR</td>
<td>18</td>
<td>16</td>
<td>15</td>
<td>83</td>
<td>93</td>
</tr>
<tr>
<td>Partial remission</td>
<td>66</td>
<td>52</td>
<td>28</td>
<td>42</td>
<td>54</td>
</tr>
<tr>
<td>Stable disease</td>
<td>22</td>
<td>20</td>
<td>11</td>
<td>50</td>
<td>55</td>
</tr>
<tr>
<td>Primary refractory</td>
<td>14</td>
<td>8</td>
<td>5</td>
<td>36</td>
<td>63</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>25</td>
<td>14</td>
<td>6</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td>Relapse</td>
<td>14</td>
<td>9</td>
<td>6</td>
<td>43</td>
<td>67</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>121</td>
<td>72</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

---

**Table 3. Complete Remission Following BMT by Stage at Diagnosis**

<table>
<thead>
<tr>
<th>Stage at Diagnosis</th>
<th>Total No. of Patients</th>
<th>No. of Evaluated Patients</th>
<th>CR Following BMT</th>
<th>% of Total</th>
<th>% of Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>22</td>
<td>19</td>
<td>15</td>
<td>68</td>
<td>79</td>
</tr>
<tr>
<td>II</td>
<td>30</td>
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<td>11</td>
<td>37</td>
<td>61</td>
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<tr>
<td>III</td>
<td>109</td>
<td>83</td>
<td>45</td>
<td>41</td>
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<td>Unknown</td>
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<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>121</td>
<td>72</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

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**Table 4. Complete Remission Following BMT by Subtype**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total No. of Patients</th>
<th>No. of Evaluated Patients</th>
<th>CR Following BMT</th>
<th>% of Total</th>
<th>% of Evaluated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>80</td>
<td>58</td>
<td>27</td>
<td>34</td>
<td>47</td>
</tr>
<tr>
<td>IgA</td>
<td>33</td>
<td>25</td>
<td>17</td>
<td>52</td>
<td>68</td>
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<tr>
<td>Light chain</td>
<td>31</td>
<td>23</td>
<td>18</td>
<td>58</td>
<td>78</td>
</tr>
<tr>
<td>Other</td>
<td>18</td>
<td>15</td>
<td>10</td>
<td>56</td>
<td>67</td>
</tr>
<tr>
<td>Total</td>
<td>162</td>
<td>121</td>
<td>72</td>
<td>44</td>
<td>60</td>
</tr>
</tbody>
</table>

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**Table 5. T-Cell Depletion and GVHD**

<table>
<thead>
<tr>
<th>GVHD Prevention</th>
<th>Total No. of Evaluated Patients</th>
<th>Grade of GVHD (% of evaluated patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell depletion</td>
<td>46</td>
<td>Absent I II III IV</td>
</tr>
<tr>
<td>without</td>
<td>48</td>
<td>35 13 0 4</td>
</tr>
<tr>
<td>other treatment</td>
<td>11</td>
<td>37 26 7 5</td>
</tr>
<tr>
<td>No T-cell depletion</td>
<td>88</td>
<td>32 31 26 7</td>
</tr>
<tr>
<td>Total evaluated</td>
<td>134</td>
<td>37 32 22 4</td>
</tr>
</tbody>
</table>
ceived three or more lines of treatment ($P = .02$) (Fig 6). There was also a tendency for better survival in patients who had received only one line of treatment as compared with those who had received two lines of treatment ($P = .24$). The status at conditioning was of importance. Patients who were in complete remission at the time of conditioning had significantly better survival than other patients ($P = .05$). However, within the groups partial remission, stable but nonresponsive disease, primary refractory dis-

Fig 2. Actuarial survival after BMT according to patient sex. Kaplan-Meier curves show significantly better survival among females than among males ($P = .04$).
ease, and relapse or progressive disease, there was no significant difference.

The time from diagnosis to transplant was not of significant importance for survival. However, there was a tendency for patients who were transplanted later than 6 months from diagnosis to do worse than those who were transplanted before 6 months from diagnosis. This tendency for poorer survival was weak, but strongest for
patients who were transplanted later than 36 months from diagnosis ($P = .18$).

Bone marrow transplantation procedural factors of importance for prognosis could not be detected. However, the wide variety of regimens used made comparisons between different types of regimens difficult. The number of patients in each treatment group was small, so differences were unlikely to be detected. TBI plus cyclophos-
phamide was the most commonly used conditioning method. There was no significant difference between this method and busulphan plus cyclophosphamide ($P = .24$). Although graft-versus-host prevention methods that included T-cell depletion resulted in fewer patients with GVHD grade II to IV and a greater fraction of patients without GVHD, this did not translate into a trend for better survival.

Postengraftment factors were also important predictors of survival. Patients who entered a complete remission following BMT had significantly longer survival than those who engrafted, but did not enter complete remission ($P = .001$) (Fig 7). The median survival duration for patients who were in complete remission following BMT was 60 months, and one patient in this group had survived for 10 years.

Patients who had GVHD grade III or IV had extremely poor survival. It was significantly poorer than for those who had grade I or II GVHD ($P = .02$). There was no significant difference between patients who had no GVHD and those who had grade I or II.

**Multivariate Analysis**

Multivariate analysis was attempted to estimate the significance and order of each risk factor. However, the material was probably too small for a fair analysis. No single pre-BMT factor came out as a significant risk factor, although there was a tendency for a higher risk for all of the factors that were significant in the univariate analysis. The strongest tendency for favorable survival in the multivariate analysis was female sex as opposed to male sex ($P = .07$) and being in stage I at diagnosis as opposed to being in other stages ($P = .11$). Of posttreatment prognostic factors, the most important adverse one was to have acute GVHD grade III or IV ($P = .0006$). Most important was the comparison between patients who entered a complete remission following BMT and those who did not. For fair comparison, only those patients who were not in complete remission before BMT, but entered complete remission following BMT, were compared with those who engrafted after BMT, but did not enter remission after BMT. Even then, patients who entered complete remission had significantly better survival ($P = .01$). The 5-year survival rate of patients who entered complete remission was 52% and the 7-year survival rate 47% (Fig 7).

**Relapse Rate and Relapse-Free Survival**

The relapse-free survival rate of patients who entered complete remission was 34% at 6 years (Fig 8). Nine patients are still in complete remission more than 4 years following transplantation. The overall relapse rate was 45% at 60 months (Fig 9). There was no significant difference between assessable patients who had received T-cell depletion or no T-cell depletion.
Causes of Death

At the time of analysis, 57 patients were alive and 103 had died. Two patients were lost for follow-up evaluation. The causes of death were mainly the same as those in other patients with hematologic disorders who have undergone BMT. The primary cause of death was the original disease in 27 patients, while in the others it was transplant-related, i.e., bacterial or fungal infections in 19,
show in this study, as has been previously shown for BMT in leukemias, that patients with GvHD grade I or II had a lower relapse rate than patients with no GvHD. Nor was it possible to show that T-cell depletion resulted in a higher relapse rate, as has previously been shown for chronic myelocytic leukemia. However, it is possible that the heterogeneity in the present study has prevented the detection of such a possible graft-versus-myeloma effect, which would have been abolished or diminished by T-cell depletion.

The most important predictor for long-term survival following BMT was complete remission after engraftment. The difference between survival of patients who entered remission and patients who engrafted with signs of multiple myeloma was highly significant. Also, a substantial fraction of complete responders are still in complete remission up to 10 years following transplantation. Thus, obtaining a complete remission is crucial for long-term survival. Persistence of myeloma cells in serum, or light chain in urine will probably inevitably result in disease progression. However, a sizeable fraction of patients with very poor prognostic parameters before transplantation survived for more than 2 years, although they did not enter complete remission. Thus, it is possible that BMT may prolong life even in a fraction of patients who do not enter remission following transplantation.

This prognostic factor analysis does not give conclusive help in selecting patients for allogeneic BMT. Since the best results are obtained in females, in patients who are diagnosed in stage I irrespective of whether they are transplanted in another stage, in patients who have received only one line of treatment, and in those who were in complete remission already before transplantation, such patients appear to be reasonable candidates. However, as these groups of patients may also have relatively good prognosis with other treatment modalities, such as ABMT, with a lower initial transplantation mortality, it is difficult at this stage to recommend allogeneic BMT unreservedly for all of these patient groups. Still, it seems reasonable to conclude that allogeneic BMT could be performed at a stage when first-line treatment fails or if the patient is unresponsive to first-line treatment. In such cases, survival with chemotherapy is usually poor. Also, BMT can be performed not only in patients less than 40 years of age but also in older patients, probably up to 55 years of age. Although factors that predict poor prognosis with conventional chemotherapy, such as stage III multiple myeloma and a high β2-microglobulin level, also predict for relatively poor prognosis with transplantation, some patients might be candidates for BMT, since long-term survival is sometimes obtained. Patients with IgA myeloma seem to be especially good candidates for BMT.

The choice of treatment modality in multiple myeloma is becoming increasingly complex. Chemotherapy, ABMT, and allogeneic BMT are now competing methods. Although our results indicate that allogeneic BMT should preferably be done early, perhaps during or after failure of first-line treatment, and before several lines of treatment, comparative studies must be performed to determine the value of each treatment method. Controlled, prospective trials are extremely difficult to perform. For that reason, matched-pair analysis studies, as has been done for comparison of BMT and chemotherapy in acute leukemia, may be more realistic to delineate factors that might guide selection of one or the other method. Such analyses are in progress.

APPENDIX

The following centers participated in the study by reporting patients to the Myeloma Registry at Huddinge Hospital Hospital: Centre Hospitalier Regional et Universitaire, Angers, France (M. Boasson); Hospital Clinic, Barcelona, Spain (J. Bladé, C. Rozman); Kantonshospital, Basel, Switzerland (A. Gratwohl); Hospital Jean Minjoz, Besançon, France (M. Fesch); Hospital San Orsola, Bologna, Italy (M. Cavo, S. Tura); Institut Jules Bordet, Brussels, Belgium (L. Debuysche); Cliniques Universitaires St. Luc, Brussels, Belgium (A. Ferrant); Hospital Casen, Cenon, France (X. Troussard); Grooto Shuur Hospital, Cape Town, South Africa (P. Jacobs); Hospital Henri Mondor, Creteil, France (J.-P. Vernant); Universität Düsseldorf, Düsseldorf, Germany (K. Quackeck); Ospedale di Careggi, Firenze, Italy (F. Rossi); Hospital Cantonal Universitaire, Geneva, Switzerland (B. Chapuis); Ospedale San Martino, Genova, Italy (M. Van Linn); Hospital A. Michallon, Grenoble, France (C. Chabannon, M. Michallet); Medical School of Hannover, Hannover, Germany (H. Link); University of Helsinki, Helsinki, Finland (L. Völl); Huddinge Hospital, Huddinge, Sweden (G. Gahrton, P. Jangman); Christian-Albrechts-University, Kiel, Germany (N. Schmitz); University Hospital, Leiden, The Netherlands (van de Loos); Hospital Claude Huriez, Lille, France (T. Fonc); University Medical Center, Ljubljana, Slovenia (J. Prentar); Royal Marsden Hospital, London, United Kingdom (P. Selby); The London Clinic, London, United Kingdom (P. Greaves); Royal London Hospital, London, United Kingdom (A.C. Newland); Charing Cross Hospital, London, United Kingdom (D. Samson); University Hospital, Lund, Sweden (B. Sallerfors); Hotel Dieu, Nantes, France (J.L. Harousseau); University Hospital, Nijmegen, the Netherlands (T. de Witte, A. Schattenberg); Hospital Cochin, Paris, France (C. Belanger); Pesaro Hospital, Pesaro, Italy (G. Lacarrèri); Hospital du Haut Leveque, Pessec, France (J. Reiffers); S. Camillo Hospital, Roma, Italy (A. De Laurenti); Dr Daniel Den Hoed Cancer Center, Rotterdam, the Netherlands (A. Hagenbeek); University Central Hospital, Turku, Finland (J. Nikoskelainen, A. Toivanen); and University Hospital, Utrecht, the Netherlands (L. Verdonck).
REFERENCES


Allogeneic and Syngeneic Hematopoietic Stem Cell Transplants in Patients With AL-Amyloidosis: A Report From The European Group For Blood And Marrow Transplantation (EBMT).

AL amyloidosis is caused by a plasma cell dyscrasia in which clonal immunoglobulin light chains deposited in tissues leads to organ failure and death. Treatment with high dose melphalan and autologous PBSC rescue produces hematologic remissions in approximately 40% of evaluable patients and improvements in organ disease and quality of life. For a subgroup of patients, an allogeneic hematopoietic cell transplantation (HCT) may be useful. There is little experience with this procedure in patients with AL amyloidosis. We report the patients (pts) who were registered within the EBMT database. Nineteen pts (median age 47 years, range 30-63; 8 female) with AL amyloidosis (n=17) or with multiple myeloma plus AL amyloidosis (n=2) underwent allogeneic (n=16) or syngeneic (n=3) HCT during 1987-2003, 11 of them before 2000. The following analysis was performed for allogeneic cases. Donors of allogeneic transplants were matched (n=13) or mismatched (n=1) related siblings or unrelated donors (n=2). Main organ manifestations were kidney, heart and liver. Indications for allogeneic HCT were young age, relapse after autologous HCT and progressive or refractory disease. Conditioning regimen was ablative in 8 pts and reduced-intensity conditioning (RIC) was used in the remaining 8 pts. Six pts had in-vivo or in-vitro T cell depletion. Engraftment was documented in 14/16 pts, 1 pt rejected the graft and 1 pt died before documented engraftment. Seven pts developed acute GVHD, 1 pt grade I, 4 pts grade II, 2 pt grade III/IV. Six of 10 evaluable pts developed chronic GVHD (4 pts limited, 2 pts extensive disease). Nine pts (56%) died at a median of 83 (range 25 - 1253) days after HCT, 5 deaths occurred before day +100. Causes of death were: cerebral aspergillosis (n=1), cardiac arrest (n=5), EBV lymphoma (n=1), gastrointestinal bleeding (n=1), disease progression (n=1), pneumococcal sepsis (n=1). Four deaths were TRM, 4 pts died of amyloidosis complications (3 cardiac deaths) and in one pt it can not be assigned. Seven pts remain alive (median observation 31 months, range 10-79) after HCT. Best hematological responses after HCT in surviving pts were immunofixation-negative CR in 6/7 pts (not available in 1 pt). In summary, a high day +100 mortality was observed. Main problem was cardiac failure due to amyloidosis or in combination with transplant-associated complications in the early phase after HCT. Of note, complete hematological responses can be achieved followed by organ response. This leads to a long-term survival of 44% in relapsed or refractory patients with AL amyloidosis after allogeneic HCT. Whether RIC plays a more important role for this patient group in the future remains to be defined within a prospective clinical study.

EBMT Annual Meeting, 20 - 23 March, 2005

EBMT Annual Meeting
20 - 23 March 2005,
Prague, Czech Republic
Allogeneic and syngeneic hematopoietic cell transplantation in patients with amyloid light-chain amyloidosis: a report from the European Group for Blood and Marrow Transplantation

Stefan O. Schöfl, Henk Lokhorst, Agnes Buzyn, Veronique Leblond, Ute Hegenbart, Giuseppe Bandini, Andrew Campbell, Enric Carreras, Augustin Ferrant, Leanne Grommich, Peter Jacobs, Nicolaus Kröger, Giorgio La Nasa, Nigel Russell, Pierre Zachree, Hartmut Goldschmidt, Simona Iacobell, Dietger Niederwieser, Gisela Galton, for the Chronic Leukemia Working Party (CLWP), Myeloma Subcommittee of the European Cooperative Group for Blood and Marrow Transplantation (EBMT)

Using the European Group for Blood and Marrow Transplantation (EBMT) registry, we retrospectively studied 19 patients with AL amyloidosis (Allo; n = 15) or syngeneic (Syn; n = 4) hematopoietic stem cell transplantation (SCT) between 1991 and 2003. For allo-SCT, full-intensity conditioning was used in 7 patients and reduced-intensity conditioning (RIC) in 8 patients. Engraftment was durable in 12 of those 15 patients. The median follow-up time is 19 months. Kaplan-Meier probabilities of overall and progression-free survival were 60% and 53% at 1 year, respectively. Overall, 40% of patients died of transplant-related mortality (TRM). Best hematologic response after SCT was complete remission (CR) and partial remission (PR) in 8 and 2 patients, respectively, leading to an organ response in 8 of these patients. Seven of the 10 patients in remission are long-term survivors. In 5 of 7 evaluable patients in CR, chronic graft-versus-host disease (GVHD) was observed, indicating the contribution of immune effects to disease control. The main clinical problem was cardiac failure in patients with poor performance status due to amyloidosis or in combination with severe infections. These data suggest that allo-SCT might be a promising and potentially curative treatment modality for selected patients with AL amyloidosis. (Blood. 2005;107:2578-2584)

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Introduction

Systemic AL (amyloid light chain) amyloidosis is a protein conformation disorder caused by a clonal plasma cell dyscrasia. Symptoms result from fibrillar extracellular deposits in kidney, heart, liver, gut, peripheral nervous system, and other tissues. The deposits disrupt organ function and ultimately lead to death. The prognosis of systemic AL amyloidosis is poor; probably less than 5% of all patients survive 10 years or longer. Because it is a rare disease and basically related to multiple myeloma (MM), most of the treatment approaches have been adopted from the experiences in MM. Using conventional chemotherapy with melphalan/prednisone, the median survival was 18 months. Treatment with high-dose melphalan (HDM) and autologous stem cell transplantation (auto-SCT) can stabilize and even reverse the disease course. In 40% to 50% of patients, complete hematologic remission can be achieved, which leads to improvement of organ function in two-thirds of these patients. A case control study showed the superiority of HDM compared with alkylator-based conventional chemotherapy regimens. However, HDM causes a high transplant-related mortality (TRM) up to 43%6-8. Major progress to decrease mortality of high-dose chemotherapy has been achieved by definition of risk groups to identify patients who will not benefit from HDM.9 The treatment of patients with unresponsive disease or relapse after auto-SCT has only been evaluated in a few prospective studies so far. Thalidomide is an effective second-line therapy for MM10 and has been tested as a single agent in patients with AL amyloidosis in a phase 2 trial.11 Complete remission (CR) was not reached in any patient and 50% of patients experienced grade 3/4 toxicity. The combinations of dexamethasone-alpha interferon12 or thalidomide/dexamethasone were also used and found to be effective but rather toxic in newly diagnosed patients.13 The

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A complete list of the Chronic Leukemia Working Party's membership is provided in the "Appendix."

S.O.S. designed and performed the study and wrote the manuscript; G.G. co-designed the study and assisted in writing the manuscript; U.H. and H.G. assisted in performing the study and writing the manuscript; and M.N. co-designed the study; B.I. performed statistical analysis; H.L. (4 patients included), A.B. (3 patients included), and H.L. (2 patients included) analyzed and provided data of patients; G.B., A.C., E.C., A.F., I.S., P.J., N.K., Y.L., R.N., and P.Z. (1 patient each) analyzed and provided data of patients.

An inside blood analysis of this article appears at the front of this issue.

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feasibility of a second auto-SCT after relapse as well as tandem auto-SCT has also been tested. A few anecdotal reports on successful allogeneic SCT (allo-SCT) have raised the possibility that allo-SCT could be a curative option for patients with AL amyloidosis. Here, we review retrospectively 19 patients with AL amyloidosis reported to the European Group for Blood and Marrow Transplantation (EBMT) registry who had undergone allo-SCT or syngeneic SCT (syn-SCT).

Patients, materials, and methods

This study was conducted on behalf of the Chronic Leukemia Working Party of the EBMT. All EBMT centers report a minimal essential data set (MED-A form) into a central database. After identification of eligible patients, centers were contacted to get information about patients' history, amyloidosis manifestations, transplant course, detailed parameters of organ response, as well as hematologic remission and follow-up. Missing information and data inconsistency were clarified by individual requests. Data were stored at the Leiden EBMT Data Centre for analysis. Informed consent was obtained locally according to the regulations applicable at the time of transplantation. Approval for this study was obtained from the Institutional Review Board of the University of Heidelberg.

Definitions and diagnostic criteria

All patients have been evaluated for AL amyloidosis or MM by standard investigations. MM stage I was distinguished from AL amyloidosis if the bone marrow infiltration by plasma cells was at least 30% or monoclonal light-chain excretion in urine was greater than 1 g/dl. The diagnosis of AL amyloidosis was done by positive Congo red staining of tissue biopsies. All patients had a monoclonal gammapathy in serum and/or urine. In 16 of 19 patients, immunohistochemistry was additionally performed to confirm the AL type of amyloidosis. One further patient showed a typical clinical symptom of AL amyloidosis (nephrotic syndrome). No patient had polymyositis or PAN. In pathologic examination, there were no signs of a plasma cell dyscrasia and positive Congo red staining. Performance status (PS) was assessed according to World Health Organization criteria (good: PS < 2; poor: PS > 2). In our study, the definition of patients with high risk for TRM were age older than 50 years at SCT using matched unrelated donors or older than 55 years using matched related donors, poor PS, more than 2 organs involved, New York Heart Association classification stage more than II, and previous auto-SCT (excluding patients with a planned auto-allo approach). The definition of reduced-intensity conditioning (RIC) was determined by contributing centers and based on the current EBMT guidance. Toxicity was analyzed with National Cancer Institute Common Toxicity Criteria (CTC) version 2.0.

Hematologic response, relapse, and disease progression were defined according to published MM criteria. In all patients surviving to at least day +100, and organ response was evaluated as early as 3 months after allo-SCT as published. Overall survival (OS) was measured in months and defined as the time from the date of transplantation to the date of death or last follow-up. Progression-free survival (PFS) was defined as the time from transplantation until date of progression or death from any cause or last follow-up. TRM was defined as death due to any cause other than disease progression or relapse occurring at any time after transplantation.

Graft-versus-host disease grading and therapy

The local investigators used standard criteria to grade acute and chronic graft-versus-host disease (GvHD). Treatment of acute and chronic GvHD was per each institution's standard practice guidelines.

Chimerism analysis

Chimerism analysis was performed per each institution's standard practice guidelines.

Statistical analysis

OS and PFS were estimated by the Kaplan-Meier method, and TRM and disease-related mortality were summarized using cumulative incidence estimates. Data were analyzed as of January 25, 2005. The package CMPSR (by R. Gray; version 2.1-1, 2002; run on R, version 1.6.2) was used for the computation of cumulative incidence curves.

Results

Patient characteristics

Between 1987 and 2005, 20 patients with AL amyloidosis who underwent allo-or syn-SCT have been reported to the EBMT. Treatment centers are listed in the "Acknowledgments." One patient who received a transplant in 1987 had to be excluded from further analysis because of an incomplete data set. Four of the cases had been published as case reports and were updated for this analysis (UPN 23923221; 24921761; 71794541; UPN 6092762). Pretransplantation patient characteristics are listed in Table 1. Of 19 patients with AL amyloidosis (median age 47 years; range, 30-63 years), 13 patients had AL amyloidosis with monoclonal gammopathy and 6 patients had MM with symptomatic AL amyloidosis. All 4 MM patients classified as stage I had a single diagnostic criterion a monoclonal light-chain excretion in the urine greater than 1 g/dl. Chromosomal analysis has been performed in 8 patients: 5 patients had a normal karyotype, 2 patients had deletion of chromosome 13 (UPN 16092762 and 1600231), and 1 patient showed deletion of chromosome 21 (UPN 23925391). Four patients underwent syn-SCT. Fifteen patients received an allo-SCT and are described in detail in this paper.

Indications for allo-SCT defined by the treating physicians were "younger age" and relapsed or refractory disease. Dominant organ affection at SCT was mainly kidney. Five patients had amyloidic cardiac disease. One patient had prior cardiac transplantation (UPN 16092762) and another patient was on dialysis at the time of SCT (UPN 1600237). The median number of organs involved was 2. The median duration from diagnosis to SCT was 9 months (range, 3-122 months). PS was graded as poor in 4 patients.

Pretreatment and conditioning regimen

Four patients were untransplanted. Eight patients had been treated with conventional chemotherapy and 3 patients with HDM and autologous SCT as front-line treatment. 2 of them followed directly by the allo-SCT (UPN 1600231; 81100832). Another patient had received HDM and auto-allo-SCT as salvage therapy (UPN 33958217). Altogether, 7 patients had been treated with HDM and auto-SCT. The median time interval between auto- and allo-SCT was 10 months (range, 2-30 months). Overall, only 4 patients were responsive to chemotherapy, but no patient reached CR before allo-SCT. The conditioning regimens are shown in Table 2.

Donors and graft composition

Donors of stem cells were HLA-identical siblings in 12 cases, a mismatched related sibling in 1 case, and matched unrelated voluntary donors in 2 cases. Six patients had received bone marrow and 9 patients peripheral stem cell grafts. In 4 patients, grafts had
Table 1. Patient characteristics prior to allo- or syn-SCT

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<th>UPI allogeneic</th>
<th>Diagnosis</th>
<th>Age at dx, y</th>
<th>Type of paraprotein</th>
<th>Dominant organ affected*</th>
<th>Other organs involved*</th>
<th>No. Involved organs*</th>
<th>Conventional chemo</th>
<th>Auto-SCT/neo-auto-SCTs</th>
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</table>

dx indicates diagnosis: Non-RIC, non-reduced-intensity conditioning; VMCP, vincristine/mercaptoanhydrolcyclophosphamide/prednisone; BM, bone marrow; MRD, matched related donor; PNP, peripheral blood; MUD, matched unrelated donor; C-VAMP, cyclophosphamide/vincristine/adriamycin/melphalan/prednisone; VAD, vincristine/adriamycin/dexamethasone; IDM, intermediate-dose melphalan; MP, melphalan/prednisone; deza, dexamethasone; mRRC, mismatched related donor; INF, interferon alpha; and Thal, thalidomide.

*At allo- or syn-SCT.
†After heart transplantation.
‡After liver transplantation.

undergo ex vivo and in 6 patients in vivo T-cell depletion (TCI).

The median CD34+ and CD31 cell counts transplanted were 4.4 × 10⁶ cells/kg (range, 3.3 × 10⁶ to 12.4 × 10⁶ cells/kg; 11 patients evaluable) and 1.9 × 10⁶ cells/kg (range, 0.001 × 10⁶ to 9.5 × 10⁶ cells/kg; 9 patients evaluable), respectively.

Engraftment, chimerism, and acute toxicities

The posttransplantation patient characteristics are given in Table 2. Fifteen of 15 patients engrafted. After engraftment (day +28 without hematologic recovery), chimerism data were available for 10 patients. Eight patients had 100% donor cells on day +28 or +100. One patient (UPN 39090001) who was treated with RK rejected the graft and had autologous recovery. Another patient (UPN 24006691) had mixed chimerism on day +100, lost the graft 6 months after T-cell depleted allo-SCT, and showed autologous recovery as well.

The median number of days with absolute neutrophil counts (ANCs) less than 0.5 × 10⁹/L (500/μL) was 15 days (range, 0-29 days) and with platelet counts less than 20 × 10⁹/L (20 000/μL) was 7 days (range, 0-44 days). The median number of red blood cell transfusions was 6 (range, 0-34) and of platelet transfusions was 3 (range, 0-22). The patient who did not engraft until day +28 after a conventional conditioning and bone marrow SCT died from gastrointestinal hemorrhage. No further bleeding complications have been reported. For the different conditioning forms, the hematologic toxicity parameters were as follows: for conventional conditioning, ANC less than 0.5 × 10⁹/L (500/μL) for a median of 18 days (range, 17-21 days) and platelet counts less than 20 × 10⁹/L (20 000/μL) for a median of 12 days (range, 0-23 days); for RIC, ANC less than 0.5 × 10⁹/L (500/μL) for a median of 11 days (range, 0-29 days) and platelet counts less than 20 × 10⁹/L (20 000/μL) for a median of 1 day (range, 0-44 days).

We observed febrile infections in 8 patients and cytomegalovirus reactivation in 4 patients. Nonhematologic toxicity greater than grade 2 by CTC criteria was documented in 6 patients (worsening of renal function in 1 patient, renal failure leading to dialysis and cardiac death in 1 patient, cardiac arrhythmia in 1 patient, orthostatic hypotension in 1 patient, asciites in 1 patient, fatal gastrointestinal hemorrhage in 1 patient).

Graft-versus-host disease

Clinically relevant acute GVHD (grade II-IV) occurred in 6 of 14 patients at a median of 29 days (range, 15-65 days) after allo-SCT and was severe in 2 patients (grade III-IV). The patient with acute GVHD grade IV did not respond to steroids and died at day +54 due to cerebral aspergillosis. Overall, 6 of 10 evaluable patients developed chronic GVHD. 1 patient had de novo chronic GVHD, and 5 patients had preceding acute GVHD. Four patients required treatment. Three of 4 patients responded to treatment. One of these 6 patients died 4-41 months due to pneumonia acquired outside a hospital.
<table>
<thead>
<tr>
<th>UPN allogeneic</th>
<th>Year of allograft</th>
<th>Disease status at SCT</th>
<th>Conditioning</th>
<th>Ex vivo T-cell depletion</th>
<th>Engraftment</th>
<th>aGVHD/grade</th>
<th>cGVHD</th>
<th>Best hematologic remission to SCT</th>
<th>Best organ response to SCT (evaluateable organs)</th>
<th>Observation, d</th>
<th>Current status (cause of death)</th>
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<td></td>
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<td>Yes</td>
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<td>Yes2</td>
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<td>2271</td>
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<td>CR</td>
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<td>Yes1</td>
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<td>Yes</td>
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<td>No</td>
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<td>No</td>
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<td>573</td>
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<td>18</td>
<td></td>
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</tbody>
</table>

Non-RIC indicates non-reduced-intensity conditioning; NR, no response; TBI, total body irradiation; Cy, cyclophosphamide; NE, not evaluable; TRM, transplant related mortality; Mel, melphalan; Lim, limited; CR, complete response; RESP, response; PR, partial response; NA, not available; Flu, fludarabine; ATG, antithymocyte globulin; EBV, Epstein-Barr virus; PNP, Pneumocystis; AL, amyloidosis; LD, stable disease; Ext, extensive; and PROG, progression.

*Immunofluorescence negative with persistent abnormal free light-chain ratio in the serum."
Hematologic remission and organ response

Four patients were not evaluable for hematologic remission and organ response due to early death. Best hematologic remission included CR in 8 patients, partial remission (PR) in 2 patients, and nonresponse in 1 patient. Free light-chain assay in the serum was additionally used in 6 patients and confirmed CR in 5 patients. One patient had an abnormal kappa-lambda ratio, although, becoming immunofixation-negative and developing organ progression (Table 2; UPN 16092762). However, we rated this patient as still in CR because EBMT criteria were used for this analysis.

The median time from allo-SCT to CR was 3 months (range, 2-15 months). In 5 of 7 evaluable patients in CR, chronic GvHD was observed. Four of 4 evaluable patients with kappa isotype achieved CR (including the 2 patients with chromosome 13 deletion) compared with 4 of 8 patients with lambda isotype. One patient had a relapse 11 months after allo-SCT and is currently being treated with thalidomide/doxycycline. Donor lymphocyte infusions have not been performed in any patient.

Organ responses were observed in cardiac transplant and no further evaluable organ (UPN 16092762). Evaluable organ involvement is shown in Table 2.

Overall survival and treatment- and disease-related mortality

Eleven of 15 patients have to be classified as high-risk patients for TRM as described in "Definitions and diagnostic criteria." The median follow-up time of all patients is 19 months (range, 1-121 months) and of surviving patients 31 months (range, 19-121 months). Kaplan-Meier probabilities of OS and PFS were 60% (95% confidence interval [CI], 40% to 91%) and 53% (95% CI, 33% to 86%) at 1 year and 52% (95% CI, 32% to 86%) and 46% (95% CI, 26% to 80%) at 2 years, respectively (Figure 1). The median OS and PFS are 42 and 19 months, respectively. The overall day +100 mortality was 27%. Three of 6 patients died before day +100 after conditioning including total body irradiation (TBI) with 8 to 12 Gy. Causes of TRM were acute GvHD grade IV and cerebral aspergillosis in 1 patient, gastrointestinal hemorrhage in 1 patient who did not engraft until day +28, and EBV-related lymphoma in the third patient, who received a transplant of an ex vivo T-cell-depleted graft. One patient died at 41 months of pneumonia associated with chronic GvHD. Another 2 patients died of cardiac failure at days +51 and +160 while suffering from kidney failure or extensive chronic GvHD. Both had cardiac involvement before allo-SCT, which probably contributed to their death. Including these patients the overall TRM was 40%. Overall disease-related mortality was 13% and estimated at 1 year at 6.7% (95% CI, 1% to 48%).

Clinical results of T-cell depletion

Four patients received an ex vivo T-cell-depleted transplant and engrafted. Two patients developed severe acute GvHD and died early (days +54 and +106). The latter patient additionally developed an EBV lymphoma. The third patient lost the graft 6 months after transplantation and is alive in PR. The fourth patient had no response and died from organ progression 19 months after SCT. None of the patients developed chronic GvHD.

In a further 6 patients, conditioning included in vivo TCD, which was used as part of RIC in 5 of those patients. Two patients died of organ progression or TRM. 1 patient is alive in relapse, and 3 patients are alive in CR about 3 years after allo-SCT.

Clinical results in 4 patients untreated before allo-SCT

Two of 4 patients untreated before allo-SCT lost their graft either after TCD or minimal conditioning with 2-Gy TBI. Despite losing the graft after 6 months, autologous reconstitution occurred in 1 patient who is in PR and is a long-term survivor. The other patient also had autologous recovery. She had a poor PS at SCT and died of TRM. The third patient received a T-cell-depleted SCT. She had no response of her myeloma, and died of organ progression. The fourth patient was a long-term survivor in CR and died of an infection related to chronic GvHD.

Syngeneic transplants

Patient characteristics are given in Table 1. Three of the 4 patients had been unresponsive to high-dose chemotherapy, 1 patient was untreated and had a previous liver transplantation. Two of the 4 patients had had a poor PS with advanced heart disease at the time of SCT and died early (days +7 and +18) of TRM (sepsis with cardiac arrest). As a noninfectious complication, nausea CTC grade III was observed in 1 patient. Two patients are long-term survivors (follow-up of +97 and +157 months), both are in CR and have organ response. Time from SCT to CR was 4 and 13 months, respectively.

Discussion

The treatment of AL amyloidosis aims at eradication of the clonal plasma cell disorder to avoid further amyloid formation and deposition. This is the first series describing allo-SCT in this disorder. Our data and a recently published case report9 suggest a potent "graft-versus-plasma-cell-dyscrasia effect" in AL amyloidosis patients.

We retrospectively evaluated the results of 15 well-documented patients who received allografts from either related or unrelated HLA-matched donors. As best hematologic remissions, 8 CR were induced by allo-SCT. After a median observation of 31 months of surviving patients, only 1 relapse occurred using the EBMT criteria. The free light-chain assay is able to detect persistent plasma cell disease with a high sensitivity25 and led to the identification of another patient with amyloidosis activity who had been considered in CR. Previously, a consensus opinion regarding remission criteria in AL amyloidosis had been published,25 including normalization of the free light-chain levels as a further CR criterion.

The importance of achieving CR for OS and organ response has already been shown in the autologous setting.3 Our data confirm...
that achievement of CR is a main predictor for long-term survival after auto-SCT as well. Currently, 7 patients are alive; 5 are in a CR between 19 and 121 months following allo-SCT with organ response. CR was probably associated with the presence of chronic GVHD. As in other hematologic diseases, our analysis shows that patients not sensitive to chemotherapy are able to achieve CR after allo-SCT. We assume that this is mainly due to immunologic effects. Due to the small patient number and heterogeneous types of conditioning used in this study, it is difficult to draw firm conclusions about the optimal time point of allo-SCT.

The CR rate in our study is encouraging and warrants further investigation. However, TRM was high with 40% and, in our opinion, several factors were contributing. First, 11 of 15 patients in our study have to be classified as high-risk patients for TRM. Therefore, the mortality of 40% is comparable to allo-SCT in other high-risk patients with malignant or nonmalignant diseases. Performance status and an important predictor for a poor PS in AL amyloidosis patients is mainly caused by cardiac involvement. Of 6 patients with poor PS at allo- or syn-SCT, 5 died of cardiac events. As a second factor, TBI with 8 to 12 Gy was associated with high day +100 mortality in our analysis. In MM and auto-SCT, TRM has mostly been deleted from conditioning regimens because of a significantly higher toxicity and lack of improvement of long-term results. In contrast, TBI-including regimens have not been proven to be inferior to chemotherapy-only regimens in allo-SCT. The importance of “full-intensity” or “reduced-intensity” conditioning remains a matter of debate. To reduce toxicity RIC could be a promising attempt in AL amyloidosis patients. Nonalpnea mortality has been reported to be significantly lower in RIC compared with conventional conditioning. The risk of severe acute GVHD is also reduced but rates of chronic GVHD have been observed to be similar. In our study, only 2 of 8 patients treated with RIC died of TRM compared with 4 of 7 treated with non-RIC. Of note, these 2 patients were in poor PS at SCT. Third, ex vivo TCD resulted in a worse clinical outcome. Comparable results have been observed in MM. In contrast, 7 of 11 patients without ex vivo TCD achieved a CR and 5 are longterm survivors. In conclusion, we believe that allo-SCT is a promising therapeutic option for patients with AL amyloidosis. In this study we observed a potent graft-versus-host-cell –lymphostasis effect. Full-intensity conditioning with TBI or ex vivo TCD were associated with an unfavorable outcome. Patients could be eligible for allo-SCT if they have not achieved a CR 6 months after HDM with auto-SCT, are still in a good PS, and have an HLA-matched donor. A prospective phase 2 study for allo-SCT using RIC is planned by the EBMT.

Acknowledgments

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We thank Anja van Biesen and Miriam van Gestel from the EBMT data registry in Leiden, The Netherlands, for their assistance with data collection.

Appendix


References


Factors influencing the risk of acute and chronic graft-versus-host disease in humans: a preliminary report from the IBMTR.


International Bone Marrow Transplantation Registry, Medical College of Wisconsin, Milwaukee.

Publication Types:
- Clinical Trial
- Comparative Study
- Multicenter Study

PMID: 2653507 [PubMed - indexed for MEDLINE]
Bone marrow transplantation from related donors other than HLA-identical siblings: effect of T cell depletion


International Bone Marrow Transplant Registry, Medical College of Wisconsin, Milwaukee, WI; University of California, Los Angeles, CA; Radiobiological Institute, Rijswijk, The Netherlands; St George's Hospital Medical School, London, England; University of KY, Lexington, KY; Universitaet Muenchen, Munich, Germany; Dr Daniel den Hoed Cancer Center, Rotterdam, The Netherlands; Center for Adult Diseases, Osaka, Japan; University of Minnesota; Karolinska Institute, Huddinge, Sweden; University of Leiden, Leiden, The Netherlands; University of Wisconsin, Madison, WI, and Prince of Wales Children's Hospital, Randwick, NSW, Australia

Summary:

Results of 470 bone marrow transplants from related donors other than genotypically HLA-identical siblings (alternative related donors) were analysed to identify factors associated with transplant outcome and to determine whether T cell depletion improved results. As compared to 3648 transplant from HLA-identical siblings, alternative related donor transplants were associated with increased graft failure, increased acute graft-versus-host disease (GVHD), and lower disease-free survival. The likelihood of adverse outcome correlated with increasing donor-recipient HLA-disparity. In multivariate analysis of alternative related donor transplants, donor age ≥30 years, (relative risk [RR] 1.7, p < 0.006), intermediate and advanced leukemia (RR 1.5 and 1.6, p < 0.01 and p < 0.003), infection pretransplant (RR 1.7, p < 0.005) and 2- and 3-locus donor-recipient HLA-disparity (RR 1.3, p < 0.04) were associated with increased risks of treatment failure. The 2-year probability of leukemia-free survival after alternative related donor transplants (n = 43) with none of these adverse prognostic features was 44% (95% confidence interval 28-59%) compared to 56% (95% confidence interval 52-59%) for similar patients receiving HLA-identical sibling transplants (n = 868, univariate p < 0.03). T cell depletion increased graft failure and decreased acute GVHD after alternative related donor transplants but did not improve leukemia-free survival.

Most include relatively few patients. Several of these reports note increased risks of severe acute graft-versus-host disease (GVHD) with alternative donor transplants. Because of this, some centers perform T cell depletion on bone marrow from HLA-mismatched donors. The results of this approach are not well-described. In this study we analysed data from 470 transplants using alternative related donors and reported to the International Bone Marrow Transplant Registry (IBMTR); 198 of these transplants (42%) used T cell-depleted marrow. Results of this analysis indicate that transplants from alternative related donors are successful in some patients and identify factors correlated with outcome.

Patients and methods

Patient population

Between 1 January 1980 and 31 December 1987, 91 centers reported data to the IBMTR for 470 patients with leukemia or aplastic anemia receiving bone marrow transplants from alternative related donors. All received methotrexate, cyclosporine, and/or T cell depletion to modify or prevent GVHD. The donor was a partially HLA-matched sibling in 295 instances, a parent in 149, and another relative in 26. Controls included 3648 patients receiving HLA-identical sibling transplants with similar prophylaxis against GVHD at the same centers during the same interval. Excluded from analysis were 85 identical twin transplants, 43 unrelated donor transplants, 152 transplants with incomplete HLA-typing data, and 143 patients receiving alternative forms of GVHD prophylaxis or no GVHD prophylaxis. Median, minimum, and maximum follow-up times were 2 years, 4 months, and 8 years, respectively.

Donor recipient HLA compatibility

Donor recipient pairs were evaluated for matching at HLA-A, HLA-B, and the HLA-D region. IBMTR reporting forms request HLA-A, B, and DR specificities of donor, recipient, and both parents of the recipient (if
available) and result of mixed lymphocyte culture (MLC) testing between donor and recipient. HLA data for all donor recipient pairs in the study and control groups were reviewed by two of the authors (R.C.A. and M.M.H.). Results of both HLA-DR typing and MLC were reported for 318 (68%) donor recipient pairs in the study group and 1863 (51%) control pairs. MLC but not DR data were reported for 138 (29%) study pairs and 1706 (47%) control pairs. DR typing but not MLC results were reported for 174 (4%) study pairs and 79 (2%) control pairs. Parental HLA-typing data, reported for 208 (44%) study patients and 1238 (34%) controls, was used to confirm donor recipient HLA-compatibility. After stratification for degree of donor-recipient HLA-compatibility (as described below), there was no difference in outcome between patients with and without parental typing.

Definitions of HLA-matching were considered with respect to reagents available when typing was performed. Transplants were classified as mismatched at HLA-A and/or B if detectable donor recipient disparity was reported, whether or not this disparity was within an HLA cross-reactive group. Donor-recipient pairs were classified as matched at the HLA-D region if: (1) matched at HLA-DR and MLC non-reactive (99 study and 1863 control patients), (2) MLC non-reactive and DR typing not performed (61 study and 1706 control patients), or (3) matched at HLA-DR and MLC testing not reported (10 study and 79 control patients). Donor-recipient pairs were considered HLA-D region mismatched if: (1) HLA-DR mismatched and MLC reactive (n = 104), (2) MLC reactive with HLA-DR typing not reported (n = 74), or (3) mismatched at HLA-DR and the MLC was non-reactive (n = 51) or not reported (n = 7).

Sixty-four donor recipient pairs in the study group were reported as matched at HLA-DR but reactive in MLC. These cases were included in the study group in univariate analyses comparing HLA-identical sibling with all alternative related donor transplants (see Table II) but excluded from most multivariate analyses examining the impact of degree of donor-recipient disparity on outcome since the degree of disparity was uncertain. They were considered as a separate group for analyses of GVHD but could not be analysed separately for graft failure, relapse and leukemia-free survival since the latter required stratification for disease and/or disease status and the numbers of patients were too small.

The degree of donor-recipient HLA-disparity for the 470 alternative related donor transplants is shown in Table I. Twenty-nine transplants were mismatched only for GVHD (homozygous recipient) and 43 mismatched only for graft rejection (homozygous donor).11,12 Unidirectional and bidirectional HLA-mismatches did not differ in outcome and were therefore combined using the overall histocompatibility assignment for each transplant. Assignment to HLA-A or B cross-reactive groups used criteria of the US National Marrow Donor Program.20

### Outcome definitions

Graft failure was analysed in patients surviving ≥ 21 days post-transplant using published criteria.21 Acute GVHD was defined as moderate to severe (grades II–IV) using published criteria.22 Patients surviving ≥ 21 days with evidence of engraftment were considered at risk of acute GVHD. Chronic GVHD was determined by clinical criteria in patients surviving ≥ 90 days with evidence of engraftment. Remission of acute leukemia was defined as absence of leukemia in any site. Relapse and remission of chronic myelogenous leukemia were defined by hematologic criteria.23

### Statistical techniques

Patient, disease and transplant-related characteristics of the study and control groups were compared using the chi² test for categorical and the Mann-Whitney test for continuous variables. Because the diseases for which transplants were performed differed significantly between study and control groups, and because the distribution of important factors such as disease status and age also differed by disease, comparisons were performed after appropriate stratification (Table II). Leukemia was classified as ‘early’ (first remission or first chronic phase), ‘intermediate’ (≥ 2nd remission or accelerated phase), and ‘advanced’ (relapse or blast phase). Variables significantly different between the study and control groups (p < 0.05) were included as covariates in all multivariate comparisons of the two groups.

Patients with leukemia and severe aplastic anemia were analysed separately for probability of graft failure and disease-free survival. Because previous IBMTR analyses do not show a difference in the risk of GVHD after transplants for leukemia and severe aplastic anemia,22,23 patients were pooled for this analysis to increase statistical power. However, use of regression models that stratified for disease and separate analyses of aplastic anemia and leukemia patients showed similar associations.

Actuarial probabilities of graft failure, acute and chronic GVHD, leukemia relapse, and disease-free survi-

### Table I: HLA-compatibility

<table>
<thead>
<tr>
<th>Alternative related donors (study group)</th>
<th>Phenotypic match (non-siblings)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One antigen mismatched</td>
<td>38</td>
</tr>
<tr>
<td>HLA-A or B</td>
<td>109</td>
</tr>
<tr>
<td>HLA-D/DR</td>
<td>139</td>
</tr>
<tr>
<td>Two antigens mismatched</td>
<td>60</td>
</tr>
<tr>
<td>HLA-A and B</td>
<td>27</td>
</tr>
<tr>
<td>HLA-A or B and HLA-D/DR</td>
<td>42</td>
</tr>
<tr>
<td>Three antigens mismatched</td>
<td>62</td>
</tr>
<tr>
<td>DR match with reactive MLC</td>
<td>64</td>
</tr>
<tr>
<td>HLA-A and B matched</td>
<td>37</td>
</tr>
<tr>
<td>HLA-A mismatched</td>
<td>9</td>
</tr>
<tr>
<td>HLA-B mismatched</td>
<td>15</td>
</tr>
<tr>
<td>HLA-A and B mismatched</td>
<td>15</td>
</tr>
</tbody>
</table>

| HLA-identical siblings (control group) | 3648                          |
Table II  Patient, disease and transplant characteristics adjusted for in multivariate analysis of leukemia patients

<table>
<thead>
<tr>
<th>ALL</th>
<th>AML</th>
<th>CML</th>
<th>SAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-ident</td>
<td>HLA-ident</td>
<td>HLA-ident</td>
<td>HLA-ident</td>
</tr>
<tr>
<td>sib donor</td>
<td>sib donor</td>
<td>sib donor</td>
<td>sib donor</td>
</tr>
<tr>
<td>ARD</td>
<td>p</td>
<td>ARD</td>
<td>p</td>
</tr>
<tr>
<td>1026</td>
<td>159</td>
<td>1168</td>
<td>136</td>
</tr>
</tbody>
</table>

**Number of patients**

<table>
<thead>
<tr>
<th>Disease status</th>
<th>ALL</th>
<th>AML</th>
<th>CML</th>
<th>SAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pretransplant</td>
<td>0.05</td>
<td>0.0001</td>
<td>0.0001</td>
<td>NA</td>
</tr>
<tr>
<td>1st CR or 1st CP</td>
<td>32%</td>
<td>23%</td>
<td>62%</td>
<td>37%</td>
</tr>
<tr>
<td>2nd CR or AP</td>
<td>47%</td>
<td>51%</td>
<td>16%</td>
<td>22%</td>
</tr>
<tr>
<td>More advanced</td>
<td>21%</td>
<td>26%</td>
<td>23%</td>
<td>17%</td>
</tr>
<tr>
<td>Patient age ≥ 20 years</td>
<td>42%</td>
<td>32%</td>
<td>68%</td>
<td>54%</td>
</tr>
<tr>
<td>Disease duration &lt;6 months</td>
<td>24%</td>
<td>23%</td>
<td>NS</td>
<td>48%</td>
</tr>
<tr>
<td>Organ impairment</td>
<td>pretransplant</td>
<td>8%</td>
<td>8%</td>
<td>NS</td>
</tr>
<tr>
<td>Allogeneic F donor</td>
<td>10%</td>
<td>25%</td>
<td>20%</td>
<td>17%</td>
</tr>
<tr>
<td>Donor age ≥ 20 years</td>
<td>45%</td>
<td>66%</td>
<td>68%</td>
<td>73%</td>
</tr>
<tr>
<td>T cell depletion</td>
<td>18%</td>
<td>43%</td>
<td>0.0001</td>
<td>17%</td>
</tr>
</tbody>
</table>

ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CML = chronic myelogenous leukemia; NA = not applicable; SAA = severe aplastic anemia; ARD = alternative related donor; CR = complete remission; CP = chronic phase; AP = accelerated phase; NS = not significant; F = female

Val were calculated using standard life-table methods and expressed as probability with 95% confidence interval (CI).29 Curves were terminated when fewer than five patients were at risk. Univariate comparison of curves was performed using the Lee-Desu statistic.29 Risks of graft failure, acute and chronic GVHD, leukemia relapse and treatment failure (relapse or death from any cause) for recipients of HLA-phenotypically identical, 1-antigen mismatched, 2-antigen mismatched and 3-antigen mismatched transplants were compared to controls using Cox proportional hazards regression models adjusting for significant differences in patient, disease and transplant characteristics between the study and control groups as described above.30 Adjusted probabilities of graft failure, acute GVHD, chronic GVHD, leukemia relapse and leukemia-free survival were calculated for T cell-depleted and non-T cell-depleted transplants using Cox proportional hazard models.

In a separate analysis of recipients of alternative related donor transplants for leukemia, the following variables were examined for their association with treatment failure in a multivariate model using Cox proportional hazards regression: patient age, donor age, allo-immune female donor, donor relationship, organ impairment pretransplant, infection at time of transplant, disease status pretransplant, interval between diagnosis and transplant, conditioning regimen, method of GVHD prophylaxis, degree of donor recipient HLA match, HLA-A or B versus HLA-D region mismatch, and year of transplant. To compare outcome using different methods of T cell depletion, techniques were grouped in three categories: (1) physical methods like elutriation or lectin separation; (2) antibodies with broad specificities like Campath 1; and (3) antibodies with narrower specificities. Variables not significantly associated with treatment failure (p > 0.05) were excluded from the regression model using a stepwise backward elimination procedure. Relative risks derived from the proportional hazards model represent the risks of treatment failure for patients with unfavorable characteristics and adjusted for effects of other variables in the model.

There were too few alternative related donor transplants for aplastic anemia for reliable multivariate analysis. Because of the multiple comparisons made, we consider only p-values < 0.01 significant. p-values between 0.01 and 0.05 are reported but should be interpreted with caution. All p-values are two-tailed and based on results of multivariate analyses otherwise specified.

## Results

### Patient characteristics

Characteristics of the study and control groups are shown in Table II. A significantly higher proportion of study versus control transplants were performed for ALL, and a smaller proportion for AML (p < 0.01). Other characteristics of the study and control groups were compared after disease stratification. Recipients of AML from alternative related donors were younger, had more advanced leukemia and longer intervals between diagnosis and transplant, were more likely to receive T cell-depleted transplants, and had older donors who were more likely to have been pregnant and/or transfused (because of the use of parents). These variables were included as covariates in all multivariate analyses comparing results of HLA-identical sibling and alternative related donor transplants.

### Comparison of HLA-identical sibling and alternative related donor transplants

**Graft failure.** Graft failure was analysed separately for patients with leukemia and aplastic anemia. In patients
with leukemia, the risk of graft failure increased with increasing HLA-disparity between donor and recipient (p for trend < 0.0001, Figure 1a). Relative risks were 2.6 (p = NS), 2.0 (p < 0.009), 7.3 (p < 0.0001), and 5.9 (p < 0.0001) for HLA-phenotypically matched, 1-locus disparate, 2-locus disparate, and 3-locus disparate transplants as compared to HLA-identical sibling transplants. There was no difference in the incidence of graft failure using donors mismatched at one HLA-A or B locus (11%, 95% CI 6–20%) versus the HLA-D region (11%, 95% CI 6–19%). Graft failures were not observed in the 12 1-antigen HLA-A or B mismatched transplants in which the mismatch occurred within a cross-reactive group; nine of 76 patients had graft failure when the mismatch was not within a cross-reactive group (univariate p = NS). For transplants mismatched at two HLA-loci, there were no differences in the risk of graft failure for different combinations of HLA-A, B and D-region mismatches.

The risk of graft failure was also increased in recipients of alternative related donor transplants for severe aplastic anemia (RR 5.0, p < 0.0001); the number of patients was too small to evaluate different degrees of HLA-disparity.

**Graft-versus-host disease.** The risk of acute GVHD increased with increasing donor-recipient HLA-disparity (p for trend < 0.0001, Figure 1b). The risk of acute GVHD after HLA-phenotypically matched and 1-antigen mismatched transplants was not significantly higher than after HLA-identical sibling transplants (RR 1.0 and 1.2, p = NS). In contrast, the relative risks of acute GVHD after 2- and 3-antigen mismatched transplants were 3.1 (p < 0.0001) and 4.4 (p < 0.0001), respectively. There was no significant difference in incidence of acute GVHD between transplants mismatched at a single HLA-A (41%, 95% CI 28–55%), HLA-B (45%, 95% CI 31–60%) or HLA-D region (42%, 95% CI 34–51%) locus. Although the risk of acute GVHD was lower if the HLA-A or B mismatch was within a cross-reactive group (23%, 95% CI 9–46%) than if not (47%, 95% CI 36–59%), this difference was not statistically significant. Among transplants mismatched at two loci, there was no difference in the incidence of acute GVHD between those mismatched at HLA-A+D or HLA-B+D (68%, 95% CI 50–82%) compared to those mismatched at HLA-A+B (69%, 95% CI 47–85%).

There was no significant difference in the risk of chronic GVHD between study and control patients at any degree of HLA-disparity even after adjusting for differences in acute GVHD (Figure 1c). After stratifying for degree of donor-recipient HLA-A and HLA-B compatibility, the probabilities of acute and chronic GVHD were similar for patients matched at HLA-DR with reactive or non-reactive MLC.

**Leukemia relapse.** The risk of leukemia relapse was similar for the study and control groups in early leukemia (Table III). Recipients of 1-locus disparate transplants for intermediate leukemia had an increased risk of relapse compared to recipients of HLA-identical sibling

---

**Figure 1** Relative risk of (a) graft failure (leukemia patients only), (b) acute graft-versus-host disease (GVHD) and (c) chronic GVHD according to degree of donor-recipient HLA-match. In all cases, the control group of HLA-identical sibling transplants is used as the reference (relative risk of 1.0) and designated by *. p values refer to the comparison between each study group and the reference group. The number of patients in each group is indicated within each bar.
transplants (RR 1.9, p < 0.01). In advanced leukemia, 2- to 3-locus disparate transplants were associated with a increased risk of relapse compared to HLA-identical sibling transplants (RR 0.3, p < 0.002).

**treatment failure.** Treatment failure was analyzed separately in patients with leukemia and aplastic anemia. The k of treatment failure (relapse or death from any cause) increased with increasing HLA-disparity between donor and recipient when transplants were done for early intermediate leukemia (p for trend < 0.001 for both, Table III). Patients receiving phenotypically identical 1-locus disparate bone marrow had similar risks of treatment failure which were less than the risk for 2- and 3-locus disparate transplants. In advanced leukemia, the k of treatment failure was not significantly higher after alternative related donor rather than HLA-identical sibling transplants (Table III).

Patients receiving 1-locus disparate transplants for leukemia had similar probabilities of leukemia-free survival whether the mismatch was for HLA-A, HLA-B, or HLA-D region. The probability of leukemia-free survival was also similar after 2-antigen mismatched transplants whether the disparity was for HLA-A+B, A-A+D region or HLA-B+D region.

Treatment failure after transplantation for aplastic anemia also increased with HLA-disparity (p for nd < 0.0001). Compared to controls, the relative risk treatment failure was 2.91 (p < 0.0001) for phenotypically matched and 1-locus disparate and 5.45 < 0.0001) for 2- and 3-locus disparate transplants, justed 2-year probabilities of disease-free survival for transplantation for aplastic anemia were 27% (95% CI 16-41%) for alternative related donor transplants and 9% (95% CI 63-71%) for HLA-identical sibling transplants.

**Comparison of T cell-depleted and non-T cell-depleted transplants**

The outcome of T cell-depleted and non-T cell-depleted transplants was compared after stratification for degree of donor-recipient HLA-match (Figure 2, Table IV).

Graft failure increased with increasing HLA-dissimilarity after both T cell-depleted and non-T cell-depleted transplants but was more likely after T cell-depleted transplants for every degree of mismatch (Figure 2a).

The probability of acute GVHD was lower after T cell-depleted than non-T cell-depleted transplants for every degree of mismatch (Figure 2b). However, the probability of acute GVHD was high (64%, 95% CI 51-75%) for 2- and 3-antigen mismatched transplants even if marrow was T cell-depleted.

Donor recipient HLA-disparity did not increase the likelihood of chronic GVHD after non-T cell-depleted transplants but did increase the likelihood of chronic GVHD after T cell-depleted transplants. The probability of chronic GVHD was significantly lower after T cell-depleted as compared to non-T cell-depleted HLA-identical sibling transplants (29%, 95% CI 25-32% versus 47%, 95% CI 45-49%, p < 0.0001), but was similar for T cell-depleted and non-T cell-depleted alternative related donor transplants (Figure 2c).

Although the risk of leukemia relapse after HLA-identical sibling transplants was higher if the donor marrow was T cell-depleted, the risk of relapse after alternative related donor transplants was similar for recipients of T cell-depleted and non-T cell-depleted grafts (Table IV).

The probability of leukemia-free survival after HLA-identical sibling transplants was lower if T cell depletion was used for early or advanced leukemia (Table IV). In contrast, leukemia-free survival was similar for non-T cell-depleted and T cell-depleted alternative related donor transplants done for early and intermediate leukemia and for 1-locus mismatched transplants done for advanced disease. Leukemia-free survival rates were marginally higher after 2- and 3-locus mismatched transplants for advanced leukemia if T cell depletion was used (Table IV).

There was no significant association between leukemia-free survival and the method used for T cell depletion.
vival after alternative related donor transplants for patients with selected profiles of prognostic features is shown in Table VI. The 43 patients with favorable prognostic features (early leukemia, a phenotypically matched or 1-antigen mismatched donor<30 years of age, and no clinically significant infection in the week prior to transplant) had a 2-year probability of survival of 44% (95% CI 28-59%). The probability of survival of 808 similar patients (early leukemia, young donor, no infection) who received bone marrow from an HLA-identical sibling was 56 (95% CI 52-59%) (univariate p<0.03).

Discussion

Results of transplants from alternative related donors for leukemia and aplastic anemia are reported by several groups. These data suggest increased risk of graft failure, acute and chronic GVHD, infection, and post-transplant lymphoproliferative disorders as compared to HLA-identical sibling transplants.

We used the large IBMTR database to examine factors affecting the outcome of alternative related donor transplants. This study confirms that these transplant result in long-term disease-free survival in some patients. However, transplants from alternative related donors were associated with increased graft failure, acute GVHD, and other transplant-related complications when compared with HLA-identical sibling transplants matched for other prognostic features. Both the risk of graft failure and acute GVHD increased progressively with increasing donor-recipient HLA disparity. The result was that leukemia-free survival was significantly lower than after HLA-identical sibling transplants. In contrast to another report, this was true even for 1-locus disparate transplants. It may be that detection of worse prognosis with minor degrees of mismatch was possible in our study because of increased statistical power associated with large numbers. Alternatively, the apparently poorer outcome after closely matched (phenotypically identical and 1-antigen disparate) alternative related donor transplants may in part be related to the variety of donor recipient matching technics used by participating teams. Since only about 70% of donor recipient pairs in the study group had both DR serology and an evaluable MLC, it is possible that some cases considered to be matched at the DR region had undetected disparity. Use of extended serotyping for DP and DQ and more sensitive molecular HLA typing methods may allow better definition of donor-recipient HLA compatibility in future analyses.

Although recipients of alternative related donor transplants had a higher risk of treatment failure than recipients of HLA-identical transplants, leukemia-free survival rates of up to 44% were observed in the best candidates for alternative donor transplants, that is in those young patients with early leukemia, in good medical condition and with lesser degrees of donor recipient HLA-disparity (Table VI).

It was postulated that T cell depletion, by decreasing

Factors associated with outcome of alternative related donor transplants

Using stepwise regression analysis, older donor age, intermediate or advanced leukemia, clinically significant infection at time of transplant, and increasing donor-recipient HLA-disparity were identified as factors predicting increased risk of treatment failure after alternative related donor transplants (Table VI). After adjustment for donor age and donor-recipient HLA-disparity, there was no difference in outcome between transplants using parental versus sibling donors, nor between T cell-depleted versus non-T cell-depleted transplants.

The actuarial 2-year probability of leukemia-free sur-
### Table IV
Adjusted 2-year probability (95% confidence interval) of relapse and leukemia-free survival (LFS) after non-T cell-depleted and T cell-depleted transplants according to disease status and degree of donor-recipient HLA-compatibility

<table>
<thead>
<tr>
<th>Event and donor recipient HLA-match</th>
<th>Non-T cell-depleted</th>
<th>T cell-depleted</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relapse-early leukemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-identical sib</td>
<td>1237</td>
<td>356</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>39</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>12</td>
<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>44</td>
<td>19</td>
<td>&lt;0.0009</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>14</td>
<td>29</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Relapse-intermediate leukemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-identical sib</td>
<td>745</td>
<td>179</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>22</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>11</td>
<td>36</td>
<td>NS</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>44</td>
<td>19</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>14</td>
<td>29</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Relapse-advanced leukemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-identical sib</td>
<td>479</td>
<td>95</td>
<td>&lt;0.0009</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>40</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>12</td>
<td>29</td>
<td>NS</td>
</tr>
<tr>
<td>LFS-early leukemia</td>
<td>1237</td>
<td>356</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>40</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>12</td>
<td>29</td>
<td>NS</td>
</tr>
<tr>
<td>LFS-intermediate leukemia</td>
<td>745</td>
<td>179</td>
<td>NS</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>22</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>11</td>
<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>LFS-advanced leukemia</td>
<td>479</td>
<td>95</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Phenotypic match or 1-antigen mismatch</td>
<td>57</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>2,3-antigen mismatch</td>
<td>14</td>
<td>12</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

*Derived from Cox proportional hazards regression adjusting for patient and donor age

**p** for comparison of non-T cell-depleted and T cell-depleted transplants


### Table V
Factors significantly associated with leukemia-free survival after bone marrow transplantation using alternative related donors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Favorable</th>
<th>Unfavorable</th>
<th>Relative risk of treatment failure</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disease status pretransplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st CR or CP</td>
<td>1.5</td>
<td>1.5</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>1st CR or CP</td>
<td>1.6</td>
<td>1.6</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td><strong>Donor age</strong></td>
<td>&lt;30 years</td>
<td>( \geq ) 30 years</td>
<td>1.4</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Presence of clinically significant infection at time of transplant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.7</td>
<td>1.7</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.3</td>
<td>1.3</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td><strong>Degree of donor-recipient HLA-disparity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenotypic identity or 1 locus disparity</td>
<td>2 or 3 locus disparity</td>
<td>1.3</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

CR = complete remission; CP = chronic phase; AP = accelerated phase

### Table VI
Actuarial 2-year probability (95% confidence interval) of leukemia-free survival after alternative related transplants for patients with selected prognostic features

<table>
<thead>
<tr>
<th>Donor characteristics</th>
<th>Phenotypic match or 1-antigen mismatch</th>
<th>2-3 antigen mismatch</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disease characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Donor age</strong>&lt;30 years</td>
<td><strong>Donor age</strong> ( \geq ) 30 years</td>
<td><strong>Donor age</strong> &lt;30 years</td>
</tr>
<tr>
<td>Early leukemia</td>
<td>44 (28 59%) (n=43)</td>
<td>16 (8 31%) (n=45)</td>
</tr>
<tr>
<td>Intermediate or advanced leukemia</td>
<td>28 (18 41%) (n=64)</td>
<td>10 (4 24%) (n=45)</td>
</tr>
</tbody>
</table>

*Excludes patients with clinically significant infection in the week prior to transplant
GVHD, might improve disease-free survival following alternative related donor transplants. In this study, acute GVHD was reduced by T cell depletion, but graft failure was increased. Disease-free survival was not improved. There was a significant increase in relapse rates for the alternative related donor transplants with T cell depletion. This contrasts with HLA-identical sibling transplants where leukemia relapse is significantly increased by T cell depletion. This may be because the incidence of chronic GVHD, a major factor in GVHD-related anti-leukemia effects,

\[41,42\] was not significantly different between T cell-depleted and non-T cell-depleted alternative related donor transplants. Multivariate analysis of alternative related donor transplants in leukemia also showed no significant difference in the probability of treatment failure or leukemia-free survival for individuals receiving T cell-depleted transplants. Different methods of T cell depletion appeared to produce similar results.

Increasing donor age, advanced leukemia, infection pretransplant, and increasing donor recipient HLA disparity were significantly associated with increased risks of treatment failure in alternative related donor transplants. Given the strong influence of donor recipient HLA-matching on outcome, the possible effect of mismatching for potentially 'immunodominant' HLA loci or combinations of loci is of concern.\[43\] Within the statistical power of this analysis, however, no differences in survival or in the incidence of graft failure or GVHD were found between transplants with single HLA-A, B, or D locus disparities. A potential advantage of matching for HLA and antigens within 'cross-reactive groups' has also been proposed.\[18\] No significant difference in graft failure or GVHD could be found for transplants disparate for a single HLA-A or B antigen whether or not the disparity was within a cross-reactive group. Elucidation of the impact of such HLA-matching considerations may require more patients or studies in which heterogeneity for other risk factors is controlled. New molecular HLA typing methods that determine HLA Class I\[13,31\] and Class II\[31,38\] polymorphisms may also help better define donor recipient HLA compatibility and help in selecting the most compatible donor.\[45\]

Analysis of alternative related donor transplants for aplastic anemia also demonstrated progressive risks associated with increasing HLA-disparity. Similar results are reported by others in smaller series.\[13,15,31\] There were too few patients for meaningful analysis of other factors associated with transplant outcome in this setting.

Data from this study should help to plan alternative donor transplants. The adverse effects of advanced leukemia suggest that if patients are to be considered for alternative related donor transplants, it should be sufficiently early in the disease course for a favorable outcome. T cell depletion did not improve outcome. Whether new T cell-depletion strategies will succeed requires further study. Likewise, whether transplantation from an HLA-identical or closely matched unrelated donor\[16,39\] may be preferable to that from a highly HLA-mismatched related donor also requires further study. The information in Table VI may help physicians advise potential recipients of alternative donor transplants of the likelihood of success and help in their selection between alternative treatment strategies. In some disease settings, clinical results may be superior with autologous marrow grafting,\[31,33\] intensive chemotherapy, or other non-transplant approaches.

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Graft Failure Following Bone Marrow Transplantation for Severe Aplastic Anemia: Risk Factors and Treatment Results

By Richard E. Champlin, Mary M. Horowitz, Dirk W. van Bekkum, Bruce M. Camitta, Gerald E. Elfenbein, Robert Peter Gale, Eliane Gluckman, Robert A. Good, Alfred A. Rimm, Ciril Rozman, Bruno Speck, and Mortimer M. Bortin

Graft failure was analyzed in 626 patients receiving allogeneic bone marrow transplants from HLA-identical sibling donors as treatment for severe aplastic anemia. Sixty-eight (11%) had no or only transient engraftment. Second bone marrow transplants were successful in achieving extended survival in 16 of 27 patients with transient initial engraftment but in none of ten patients with no sign of engraftment after the first transplant. The major factors associated with a reduced risk of graft failure were use of radiation for pretransplant immunosuppression and use of cyclosporine rather than methotrexate or T-cell depletion of the donor bone marrow for prophylaxis against graft-versus-host disease (GVHD). Among 266 patients prepared for transplantation with cyclophosphamide alone, the risk of graft failure was increased in patients who received previous transfusions and reduced in those who received corticosteroids for previous therapy. Neither cell dose nor administration of donor buffy coat cells affected the probability of engraftment. Although use of radiation in conditioning reduced graft failure, survival was not improved. Posttransplant treatment with cyclosporine and avoidance of pretransplant blood transfusions were associated with improved survival.

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Bone marrow transplantation is an effective therapy for severe aplastic anemia and is generally considered the preferable treatment for young patients who have an HLA-identical sibling donor. Recent studies report 55% to 80% extended survival.

Graft failure owing to rejection and other causes remains an important, life-threatening complication following allogeneic bone marrow transplantation for aplastic anemia. It occurs in 5% to 60% of patients receiving HLA-identical transplants and various pretransplant and posttransplant immunosuppressive therapies. Factors associated with graft failure and the efficacy of various immunosuppressive regimens in preventing this complication were investigated in this study.

MATERIALS AND METHODS

Results of allogeneic bone marrow transplantation were analyzed in patients with severe aplastic anemia receiving bone marrow transplants at 98 centers worldwide from January 1978 through December 1986 and reported to the International Bone Marrow Transplant Registry. Six hundred twenty-five of 657 consecutive patients who received transplants from HLA-identical sibling donors and who survived ≥21 days were considered evaluable for engraftment. An additional 19 patients who received transplants from identical twins were analyzed separately. Criteria for the diagnosis of severe aplastic anemia have been previously described.

Graft failure was defined as either (a) primary graft failure, i.e., absence of hematologic recovery in patients surviving ≥21 days posttransplant; or (b) transient engraftment, i.e., complete or partial recovery of hematopoiesis of donor origin followed by recurrent pancytopenia with a markedly hypocellular bone marrow in the absence of moderate to severe acute graft-versus-host disease (GVHD).

Statistical analysis. Actual probability of graft failure was calculated using standard life-table methods. Life-table curves were terminated when fewer than three patients were at risk of graft failure. Univariate analyses were used to test associations between patient and treatment variables and the probability of graft failure using the Lee-Desu statistic. Factors associated with the risk of graft failure in these univariate analyses with a P value ≤.10 were entered into a multivariate Cox proportional-hazards model using a forward stepwise approach. Variables significantly associated with the probability of graft failure in multivariate analysis were examined for their association with survival using a Cox proportional-hazards model adjusted for patient age. Because of the multiple comparisons made, only variables which improved the model with \( P < .01 \) were considered statistically significant. \( P \) values between .01 and .05 were considered marginally significant and are presented to show trends. Relative risks of graft failure and mortality for patients with unfavorable compared to those with favorable risk factors are derived from the multivariate models and are adjusted for the effect of all other significant variables. Relative risks for mortality are also adjusted for patient age. Because of the very low incidence of graft failure observed in irradiated patients, separate univariate and multivariate analyses were performed on the 266 patients who received cyclophosphamide alone for conditioning.

From the International Bone Marrow Transplant Registry, Department of Medicine, and the Division of Hematology/Oncology and Biostatistics/Clinical Epidemiology, Medical College of Wisconsin, Milwaukee, and the Division of Hematology/Oncology, UCLA Center for the Health Sciences, Los Angeles; Division of Health Research TNO, Radiobiological Institute, Rijswijk, The Netherlands; Department of Pediatrics, Children's Hospital of Wisconsin, Milwaukee, Division of Medical Oncology and Bone Marrow Transplantation Program, University of Florida College of Medicine, Gainesville; Service d'Hematologie, Hopital Saint-Louis, Paris; Cancer Research Program, All Children's Hospital, St Petersburg, FL; Escuela de Hematologia, University of Barcelona, Spain; and Kantonsspital, Basel, Switzerland.

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Unless otherwise specified, all P values are based on the results of the multivariate analyses.

All multivariate analyses were examined for a possible center effect, ie, differences among centers not explained by identifiable patient and treatment differences using the following methods: entering transplant team into the regression model as a categorical covariate; stratifying the regression model by transplant center; repeating the analysis after exclusion of each of the seven largest centers; and dividing patients according to whether they were transplanted in centers reporting <20 and those reporting >20 patients.  The relative risks and P values associated with each of the prognostic variables were similar with and without these adjustments.

RESULTS

Characteristics of patients, donors, and treatments. Data from 384 males and 241 females surviving ≥21 days after transplant were analyzed for factors associated with graft failure. Pretransplant patient characteristics are summarized in Table 1. Median age was 19 years (range 1 to 56 years) for recipients and 20 years (range 1 to 59 years) for their HLA-identical sibling donors. Donor and recipient age were highly correlated (r = .82). The median duration of aplasia was 2 months (range 1 to 158 months) at the time of transplantation. The etiology of aplastic anemia was idiopathic in 466 (74%) patients, drug/toxin related in 74 (12%) patients, hepatitis related in 63 (10%) patients, and associated with other causes in 22 (4%). Two hundred seventy patients were maintained in laminar airflow isolation, and 349 were maintained in conventional protective isolation.

All patients received pretransplant immunosuppressive treatment with cyclophosphamide, usually 50 mg/kg for four days; 266 patients received no additional pretransplant therapy, and 25 received additional chemotherapy such as procarbazine and/or antithymocyte globulin. Three hundred thirty-four patients received cyclophosphamide plus radiation, including 121 who received total body irradiation (TBI) 3.0 to 12.0 Gy (median 3.0 Gy), and 213 who received limited-field (total lymphoid or thoracoabdominal) radiation, 1.5 to 18.0 Gy (median 6 Gy).

Posttransplant immune suppression to prevent or modify GVHD consisted of methotrexate in 288 patients, cyclosporine in 206, cyclosporine and methotrexate in 24, methotrexate and other drugs in 50, cyclosporine and other drugs in 35, and T-cell depletion in ten, corticosteroids alone in three, and no prophylaxis in nine patients. Among recipients of T-replete transplants, the median dose of bone marrow cells was 3.3 (range 0.4 to 13.0) x 10^6/kg recipient body weight.

Graft failure. Graft failure occurred in 68 (11%) of the 625 patients with an actuarial probability of 13% ± 3% (95% confidence interval) at 5 years. Nineteen of 68 (28%) patients had primary graft failure (failed to show any sign of engraftment), and 49 (72%) patients had transient engraftment with loss of the graft 3 weeks to 3 years (median 11 weeks) posttransplant. Outcome differed for patients with no engraftment and those with transient engraftment (Table 2).

Seven of the 68 patients survived with full or partial recovery of host hematopoiesis. All seven cases occurred in the group of 49 patients who had transient engraftment after

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient age (yr)</td>
<td>19</td>
<td>1-56</td>
</tr>
<tr>
<td>Donor age (yr)</td>
<td>20</td>
<td>1-59</td>
</tr>
<tr>
<td>Interval diagnosis-transplant (mo)</td>
<td>22</td>
<td>1-158</td>
</tr>
<tr>
<td>No. of pretransplant transfusions</td>
<td>80</td>
<td>10-100</td>
</tr>
<tr>
<td>Unmanipulated cell dose (x 10^6/kg body wt)</td>
<td>3.3</td>
<td>0.4-13.0</td>
</tr>
<tr>
<td>Race</td>
<td>N/N</td>
<td>Evaluable</td>
</tr>
<tr>
<td>White</td>
<td>501/625</td>
<td>80</td>
</tr>
<tr>
<td>Non-white</td>
<td>124/625</td>
<td>20</td>
</tr>
<tr>
<td>Etiology of aplastic anemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic</td>
<td>466/625</td>
<td>74</td>
</tr>
<tr>
<td>Drug/toxin</td>
<td>74/625</td>
<td>12</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>63/625</td>
<td>10</td>
</tr>
<tr>
<td>Other</td>
<td>22/625</td>
<td>4</td>
</tr>
<tr>
<td>Infected at time of transplant</td>
<td>243/624</td>
<td>39</td>
</tr>
<tr>
<td>Previous treatment of aplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>294/617</td>
<td>39</td>
</tr>
<tr>
<td>Androgens</td>
<td>73/617</td>
<td>12</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>100/617</td>
<td>16</td>
</tr>
<tr>
<td>Androgens + corticosteroids + other*</td>
<td>129/617</td>
<td>21</td>
</tr>
<tr>
<td>Androgens + other†</td>
<td>11/617</td>
<td>2</td>
</tr>
<tr>
<td>Corticosteroids + other‡</td>
<td>19/617</td>
<td>3</td>
</tr>
<tr>
<td>Other§</td>
<td>41/617</td>
<td>7</td>
</tr>
<tr>
<td>Sex match</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M → M</td>
<td>223/625</td>
<td>36</td>
</tr>
<tr>
<td>M → F</td>
<td>116/625</td>
<td>19</td>
</tr>
<tr>
<td>F → F</td>
<td>125/625</td>
<td>20</td>
</tr>
<tr>
<td>F → M</td>
<td>161/625</td>
<td>26</td>
</tr>
<tr>
<td>Female donor alloimmunized</td>
<td>57/447</td>
<td>13</td>
</tr>
<tr>
<td>ABO match</td>
<td>379/589</td>
<td>64</td>
</tr>
<tr>
<td>Isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional isolation</td>
<td>349/624</td>
<td>56</td>
</tr>
<tr>
<td>Laminar airflow isolation</td>
<td>270/624</td>
<td>43</td>
</tr>
<tr>
<td>Radiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No radiation</td>
<td>200/624</td>
<td>46</td>
</tr>
<tr>
<td>TBI</td>
<td>12/624</td>
<td>19</td>
</tr>
<tr>
<td>Dose (Gy)</td>
<td>3.0</td>
<td>3.0-8.0</td>
</tr>
<tr>
<td>Limited-field radiation#</td>
<td>213/624</td>
<td>34</td>
</tr>
<tr>
<td>Dose (Gy)</td>
<td>8.0</td>
<td>1.5-12.0</td>
</tr>
<tr>
<td>Prophylaxis against GVHD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methotrexate alone</td>
<td>258/625</td>
<td>46</td>
</tr>
<tr>
<td>Methotrexate + other</td>
<td>50/625</td>
<td>8</td>
</tr>
<tr>
<td>Cyclosporine alone</td>
<td>206/625</td>
<td>33</td>
</tr>
<tr>
<td>Cyclosporine + other</td>
<td>35/625</td>
<td>5</td>
</tr>
<tr>
<td>Methotrexate + cyclosporine</td>
<td>24/625</td>
<td>4</td>
</tr>
<tr>
<td>T-cell depletion</td>
<td>10/625</td>
<td>2</td>
</tr>
<tr>
<td>Other</td>
<td>12/625</td>
<td>2</td>
</tr>
<tr>
<td>Buffy coat transfusion posttransplant</td>
<td>146/659</td>
<td>26</td>
</tr>
</tbody>
</table>

*Includes 24 patients who received androgens + corticosteroids + antithymocyte globulin.
†Includes ten patients who received androgens + antithymocyte globulin.
‡Includes 12 patients who received corticosteroids + antithymocyte globulin.
§Includes 14 patients who received antithymocyte globulin.
#Median.
||Range.
|##Includes total lymphoid, total nodal, and thoracoabdominal radiation.
Table 2. Clinical Outcome in 68 Allografted Patients Whose Graft Failed After the First Transplant

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Primary Graft Failure (n = 19)</th>
<th>Transient Engraftment (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive with autologous marrow recovery</td>
<td>0 (0%)</td>
<td>7 (14%)</td>
</tr>
<tr>
<td>Died of aplasia, no second transplant</td>
<td>8 (47%)</td>
<td>15 (31%)</td>
</tr>
<tr>
<td>Died of aplasia after second transplant*</td>
<td>5 (26%)</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>Died of complications of second transplant†</td>
<td>5 (26%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Alive with engraftment after second transplant</td>
<td>0 (0%)</td>
<td>16 (33%)</td>
</tr>
<tr>
<td>Three-year probability of survival (95% CI)</td>
<td>0 (± 0%)</td>
<td>46 (± 14%)</td>
</tr>
</tbody>
</table>

*Includes eight patients with no engraftment, three with partial engraftment, and two with transient engraftment after the second transplant.
†Primary causes of death after the second transplant: GVHD, three; interstitial pneumonia, three; adult respiratory distress syndrome, one; and bacterial pneumonia, one.

the first transplant; none of the 19 patients with primary graft failure had autologous marrow recovery. Twenty-four additional patients were not retransplanted, and all died of severe aplastic anemia; nine with primary graft failure died between 3 and 8 weeks (median 4 weeks) posttransplant, and 15 with transient engraftment died between one day and 7 weeks (median 2 weeks) after losing their graft.

Ten patients with primary graft failure (no engraftment) and 27 patients with transient engraftment received a subsequent transplant, 23 from the same donor and 14 from a different HLA-identical sibling donor. Thirty-four patients received two transplants, and three patients received three transplants. The median interval between the first and second transplant was 2.6 months (range 0.9 to 31 months). Prior to the second transplant, 16 patients received cyclophosphamide with or without other drugs, 14 received cyclophosphamide plus TBI, and three patients received other conditioning regimens. Four patients were retransplanted without additional immune suppression. Survival was not significantly influenced by the conditioning regimen used for the second transplant or by whether the same or a different donor was used. However, none of the ten patients retransplanted because of primary graft failure survived a second transplant. Five of the ten had full or partial engraftment but died of other transplant-related complications; the remaining five failed to engraft. Of the 27 patients who had transient hematopoietic recovery after their initial transplant and were retransplanted, 22 engrafted and 16 are alive 6 to 96 months (median 38 months) after their second transplant; their actuarial probability of survival was 59% ± 20% at 3 years.

The 3-year probability of survival for the 49 patients with transient engraftment was 46% ± 14%. None of the 19 patients with primary graft failure is alive. Overall, 37 of 68 patients with graft failure died with aplasia as the primary or a contributing cause of death for a case-fatality rate of 54%. The mortality rate owing to graft failure was 6% for the 625 patients studied.

Three patients who did not meet the study criteria for graft failure also received a second transplant. All had partial hematopoiesis in the setting of GVHD. Two died of acute GVHD and interstitial pneumonitis; one is alive with full recovery of hematopoiesis, extensive chronic GVHD, and a Karnofsky performance score of 80% ten months after the second transplant.

Risk factors for graft failure. Variables significantly associated with graft failure in multivariate analysis of the 625 patients surviving ≥21 days are shown in Table 3. It was not possible to distinguish factors associated with primary graft failure vs transplant engraftment. The most important risk factor was whether or not the preparative regimen included radiation (Fig 1A). The 290 patients who were not given radiation had an increased risk of graft failure in comparison with the 334 patients who received radiation.

Table 3. Variables Analyzed for Their Association With Graft Failure and Shown to be Significantly Associated With Graft Failure in Analysis of All Patients Transplanted for Severe Aplastic Anemia or Patients Transplanted After Conditioning With Cyclophosphamide Alone

<table>
<thead>
<tr>
<th>Variable</th>
<th>Favorable</th>
<th>Unfavorable</th>
<th>Relative Risk of Graft Failure (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditioning regimen</td>
<td>Radiation (n = 334)</td>
<td>No radiation (n = 290)</td>
<td>3.2 (1.8-5.5)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Drug to prevent GVHD</td>
<td>CsA ± other* (n = 269)</td>
<td>MTX ± other (n = 338)</td>
<td>2.1 (1.2-3.4)</td>
<td>&lt;.008</td>
</tr>
<tr>
<td>T-cell depletion</td>
<td>No T-cell depletion (n = 815)</td>
<td>T-cell depletion (n = 10)</td>
<td>10.8 (3.5-34)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Patients prepared with cyclophosphamide alone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretransplantation therapy of aplasia</td>
<td>Corticosteroids ± other drugs (n = 113)</td>
<td>Other or no treatment (n = 145)</td>
<td>2.5 (1.2-5.2)</td>
<td>&lt;.007</td>
</tr>
<tr>
<td>Pretransplantation transfusions</td>
<td>&lt;40 (n = 211)</td>
<td>≥40 (n = 51)</td>
<td>2.9 (1.5-6.8)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Drug to prevent GVHD</td>
<td>CsA ± other* (n = 122)</td>
<td>MTX ± other (n = 138)</td>
<td>2.3 (1.2-5.2)</td>
<td>&lt;.008</td>
</tr>
</tbody>
</table>

*Includes regimens containing both MTX and CsA; CI, confidence interval; CsA, cyclosporine; MTX, methotrexate.
in the 24 patients who received the combination of methotrexate and cyclosporine was similar to the risk in 206 patients who received cyclosporine alone. Although only ten patients receiving T-cell-depleted transplants were available for analysis, four had graft failure, for an actuarial rate of 47% ± 35% at 2 years (Fig 1B). The relative risk of graft failure associated with use of T-cell depletion was 10.8 (P < .001).

*Risk factors for graft failure in patients receiving cyclophosphamide alone.* Because the markedly reduced incidence of graft failure in association with preparative regimens that included radiation may have obscured findings that would help our understanding of other mechanisms of graft failure, data for the 266 patients prepared with cyclophosphamide alone were analyzed separately. Variables associated with the risk of graft failure in this group are shown in Table 3.

Two pretransplant variables were significantly associated with the risk of graft failure. Patients whose aplastic anemia was not treated with corticosteroids prior to referral for transplantation had an increased risk of graft failure as compared with those who had received corticosteroids (relative risk 2.5, P < .007, Fig 2A). Increasing numbers of pretransplant transfusions were associated with an increased risk of graft failure, with risk increasing continuously (relative risk 1.01^n, where n = number of units transfused, P < .007) and with patients who received the largest number of transfusions having the highest risk (Fig 2B). Posttransplant immunosuppressive therapy also was significantly associated with the risk of graft failure. Patients receiving methotrexate had a higher risk than those receiving cyclosporine (relative risk 2.3, P < .008, Table 3). Because only one patient in this subgroup of 266 patients received T-cell-depleted bone marrow, the effect of T-cell depletion could not be evaluated.

Among patients receiving cyclophosphamide alone for pretransplant immune suppression, the probability of graft failure decreased significantly over the course of the study. For the patients transplanted in the years 1978 through 1980, the 3-year probability of graft failure was 31% ± 12%; for patients transplanted after 1980 it was 16% ± 6% (univariate P < .005). This change coincided with a significant decrease in the use of methotrexate and a corresponding increase in the use of cyclosporine to prevent GVHD (univariate P < .0001). There was no significant change in the median number of transfusions received or the type of drugs used to treat severe aplastic anemia over this time period.

After adjustment was made for the difference in drugs used as prophylaxis against GVHD, the probability of graft failure was not significantly different in the earlier and later years of the study.

*Factors not associated with the risk of graft failure.* Factors which were not significantly associated with graft failure are presented in Table 4. Within the range available for testing, higher bone marrow cell doses were not associated with better engraftment; however, >95% of patients received > 2.0 x 10^8 cells/kg body weight. Whether cell doses lower than these would be associated with graft failure is unknown. Use of laminar airflow isolation, infusion

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Fig 1. Actuarial probability of graft failure according to (A) whether radiation was administered for pretransplant immune suppression and (B) method of prophylaxis against GVHD. *Includes patients receiving both cyclosporine and methotrexate.*

(relative risk 3.2, P < .0001). There was no significant difference in the probability of graft failure between patients receiving TBI and those receiving limited-field radiation. There was no significant difference in the probability of graft failure between limited field and TBI. There was no apparent dose effect within the range of doses reported.

The method of GVHD prophylaxis was also an important determinant of graft failure (Fig 1B). Regimens containing methotrexate were associated with a significantly higher probability of graft failure than regimens containing cyclosporine (relative risk 2.1, P < .008). The risk of graft failure
Fig 2. Actuarial probability of graft failure in patients receiving cyclophosphamide alone for pretransplant conditioning according to (A) whether corticosteroids were used to treat aplastic anemia prior to transplantation and (B) number of pretransplant transfusions.

of donor buffy coat cells, and donor-recipient sex-match did not significantly affect the probability of stable engraftment in the entire series or in the subgroup prepared with cyclophosphamide alone. Prior treatment with antithymocyte globulin was not associated with the probability of graft failure.

Relationship between risk factors for engraftment and mortality. Each of the factors significantly associated with an increased risk of graft failure was studied for its association with GVHD, interstitial pneumonia, and survival. Overall, patients treated with methotrexate rather than cyclosporine to prevent GVHD had a significantly higher incidence of interstitial pneumonia (21% vs 10%, univariate \( P < .0009 \)), and a higher risk of mortality posttransplant (relative risk 1.6, \( P < .0009 \)). The incidence of moderate-to-severe acute GVHD and chronic GVHD was similar whether methotrexate or cyclosporine was used. Only one of six patients who engrafted after transplantation with T-cell-depleted marrow developed acute GVHD; none developed chronic GVHD; two developed interstitial pneumonia. The relative risk of dying was increased after T-depleted as compared with T-replete transplants, but this was not statistically significant (relative risk 1.5, \( P > .10 \)). In comparison with irradiated patients, patients conditioned with cyclophosphamide alone had a lower incidence of moderate-to-severe acute GVHD (36% vs 45%, univariate \( P < .03 \)), a similar incidence of chronic GVHD, and a lower incidence of interstitial pneumonia (12% vs 21%, univariate \( P < .004 \)). The risk of mortality was not higher for nonirradiated as compared with irradiated patients (relative risk 0.99, \( P > .10 \)).

Among the 266 patients prepared for transplantation with cyclophosphamide alone, patients receiving methotrexate to prevent GVHD had an incidence of acute GVHD, chronic GVHD, and interstitial pneumonia similar to that of patients receiving cyclosporine. Patients receiving methotrexate had a higher risk of mortality posttransplant that was marginally significant (relative risk 1.6, \( P < .03 \)). Greater numbers of pretransplant transfusions were not associated with the incidence of acute GVHD, chronic GVHD, or interstitial pneumonia. Patients who received \( \geq 40 \) transfusions pretransplant had a higher risk of dying than those who received \(< 40 \) (relative risk 1.2, \( P < .01 \)). Pretransplant treatment with corticosteroids was not associated with GVHD, interstitial pneumonia, or survival.

Although not associated with the probability of engraftment, use of buffy coat transfusions posttransplant was associated with an increased incidence of moderate-to-severe acute GVHD (48% vs 38%, univariate \( P < .05 \)), and chronic GVHD (45% vs 36%, \( P < .07 \)), and an increased risk of mortality (relative risk 1.4, \( P < .05 \)).

Identical twin transplants. Nineteen patients received transplants from identical twin donors. Six received pretransplant immune suppression with cyclophosphamide
alone (four patients) or cyclophosphamide plus TBI (two patients). All six are alive and well with sustained engraftment 12 to 92 months after transplantation. Thirteen of the 19 patients initially received bone marrow infusion without pretransplant or posttransplant immune suppression. Four of 13 had full hematopoietic recovery and are alive 11 to 30 months after transplantation. Nine of 13 had either no (two patients) or transient (seven patients) engraftment and received a second transplant; one of the nine received a third transplant. Prior to the second transplant, all patients received immune suppression. Six received cyclophosphamide alone, two received cyclophosphamide plus radiation, and one received nitrogen mustard. Twelve of the 13 multiple transplant recipients are alive with full recovery of hematopoiesis five to 79 months after transplantation. One patient died of sepsis in the second week after the second transplant; she had full erythroid and granulocytic recovery but still had severe thrombocytopenia at the time of death.

**DISCUSSION**

This analysis examined the incidence and clinical consequences of graft failure following bone marrow transplantation for aplastic anemia and identified risk factors associated with this complication. Since it is generally not possible to discern the mechanism of graft failure in a given patient, it was operationally defined in this analysis as occurring in patients who survived >21 days but either failed to show any recovery of hematopoiesis or had engraftment manifested by partial or full hematologic recovery following recurrent aplasia in the absence of moderate to severe acute GVHD.

Frequently graft failure after HLA-identical sibling transplants is assumed to result from immunologic rejection directed at donor minor histocompatibility antigens. Other mechanisms may be responsible for graft failure as well, such as abnormalities of the recipient microenvironment, inhibition of hematopoiesis by infection, or nonimmune mechanisms. Data from identical twins reported in this article and elsewhere support the concept that host immunologic reactions against histocompatibility alloantigens on the transplanted bone marrow cells are not the sole mechanism for graft failure.

Graft failure occurred in 68 of 625 evaluable recipients of HLA-identical marrow transplants. Of 19 patients with no sign of engraftment, none survived despite attempts at further immunosuppressive preparative treatment and retransplantation in ten of the 19. The clinical condition of patients who fail to engraft is highly precarious. They tolerate further intensive immunosuppressive treatment poorly and often succumb to drug toxicity, infections, or other complications before engraftment can occur. Patients with transient engraftment have a better prognosis with second transplants; 16 of 27 are surviving six to 96 months after the second transplant, and their actuarial survival is 59% ± 20% at 3 years. Second transplants were reported by the International Bone Marrow Transplant Registry in 1976 to be successful in four of 12 (33%) patients and, in a more recent study by Storb et al, 23 in six of 16 (38%) patients with graft failure after transplantation for aplastic anemia.

Analysis of the entire group identified no pretransplant patient or donor characteristic that was significantly associated with graft failure. Various therapeutic interventions were associated with a reduced risk of graft failure, although some may lead to an increased likelihood of other complications, as shown in Table 5.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rejection</th>
<th>GVHD</th>
<th>Pneumonitis</th>
<th>Survival</th>
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<td>Radiation therapy</td>
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<td>Methotrexate rather than cyclosporine to prevent GVHD</td>
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<td>T-cell depletion</td>
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<td>Multiple transplants prior to transplant</td>
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<td>Corticosteroids pretransplant</td>
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<td>Buffy coat infusion</td>
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*Increased (I), no change (—), decreased (l).*
A trend toward a higher probability of engraftment in recent years was observed in patients conditioned with cyclophosphamide alone. This can be accounted for by the increasing use of cyclosporine to prevent GVHD. Other treatment and/or patient variables associated with this trend were not identified. Changes in blood transfusion practices or other unidentified factors may also have contributed to the reduced rate of graft failure.

Previous treatment with corticosteroids prior to referral for transplantation was associated with a lower incidence of graft failure. The explanation for this finding is unclear. The interval between diagnosis of aplastic anemia and transplantation and use of other immunosuppressive treatments (eg, antithymocyte globulin) did not appear to affect engraftment. Corticosteroids do inhibit cellular and humoral immunity in patients with aplastic anemia and thus may have facilitated engraftment by this mechanism.56

These data point to several modes of treatment associated with a reduced incidence of graft failure. In conjunction with information regarding the impact of these therapies on other transplant-related complications (Table 5), they suggest some measures that may improve the outcome of bone marrow transplantation for aplastic anemia. Cyclosporine-containing regimens appear to have an advantage over methotrexate. If possible, transplants should be withheld or minimized prior to initiation of the immunosuppressive preparative regimen, especially if regimens without radiation are to be used.

ACKNOWLEDGMENT

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APPENDIX 1

This 54th report from the International Bone Marrow Transplant Registry was prepared for the members of the Advisory Committee: Robert Peter Gale, MD, PhD, University of California, Los Angeles, Chairman; Kerry Atkinson, MD, St Vincent’s Hospital, Sydney, Australia; Fritz H. Bach, MD, University of Minnesota, Minneapolis; A John Barrett, MD, MRC Path, Westminster Hospital, London; Dirk W. van Bekkem MD, PhD, Radiobiological Institute TNO, Rijswijk, The Netherlands; James C. Biggs, MD, PhD, St Vincent’s Hospital, Sydney, Australia; Karl G. Blume, MD, City of Hope National Medical Center, Duarte, CA; Mortimer M. Bostin, MD, Medical College of Wisconsin, Milwaukee; Karel A. Dicks, MD, PhD, M.D. Anderson Hospital and Tumor Institute, Houston; Gosta Gahrton, MD Karolinska Institute, Stockholm; Eliane Gluckman, MD, Hôpital Saint-Louis, Paris; John M. Goldman, MD, Royal Postgraduate Medical School, London; Robert A, Good, MD, PhD, all Children’s Hospital, St Petersburg, FL; Werner Hobel, MD, Karl Marx University, Leipzig, DDR; Roger H. Herzig, MD, Cleveland Clinic; Richard Hong, MD, University of Wisconsin, Madison; John H. Kersey, MD, University of Minnesota, Minneapolis; Hans-Jochem Kolb, MD, University of Munich; Alberto M. Marmot, MD, Ospedale San Martino, Genoa, Italy; Tohru Masakata, MD, Center for Adult Diseases, Osaka, Japan; Hans A. Messner, MD, PhD, Ontario Cancer Institute, Toronto; Richard J. O’Reilly, MD, Memorial Sloan-Kettering Cancer Center, New York; Ray L. Powles, MD, Royal Marsden Hospital, London; Alfred A. Rimm, PhD, Medical College of Wisconsin, Milwaukee; Ole Ringden, MD, PhD, Huddinge Hospital, Huddinge, Sweden; Jon J. van Rood, MD, PhD, University of Leiden, The Netherlands; Ciril Rozman, MD, University of Barcelona, Spain; Bruno Speck, MD, University of Basel, Switzerland; Roy S. Weiner, MD, University of Florida, Gainesville; and Ferry E. Zwaan, MD, PhD, University of Leiden, The Netherlands.

APPENDIX 2

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EAST GERMANY: Karl Marx Universität, Leipzig. FRG: Christian-Albrechts Universität, Kiel; Mod. Universitätsklinik, Tubingen; Universitäts-Kinderklinik, München; Universität Ulm, Ulm/Donau; Universität Münster, Münster. HUNGARY: Semmelweis University, Budapest. INDIA: Tata Memorial Hospital, Bombay. IRELAND: St James’s Hospital, Dublin. ISRAEL: Chaim Sheba Medical Center, Tel-Hashomer. ITALY: Ospedale Riuniti di Pusaro, Pusaro; Ospedale San Martino, Genoa; S. Orsola University Hospital, Bologna; University of Milan; Università Chieti, Pescara. JAPAN: Center for Adult Diseases, Osaka; Daini Red Cross Hospital, Nagoya; Tokai University, Isehara; University of Tokyo, Minato-Ku, Tokyo. KOREA: St Mary’s Hospital, Seoul. NEW ZEALAND: Christchurch Hospital, Christchurch. POLAND: Postgraduate Medical Center, Warsaw. SAUDI ARABIA: King Faisal Hospital, Riyadh. SCOTLAND: Glasgow Royal Infirmary, Glasgow; Royal Infirmary, Edinburgh. SOUTH AFRICA: University of Cape Town Medical School, Cape Town. SPAIN: Centro Medico Nacional ‘Marques de Valdecilla,’ Santander. Clinica Puerta de Hierro, Madrid; Hospital de la Princesa, Madrid; Hospital Infantil Vall d’Hebron, Barcelona; Hospital “La Fe,” Valencia; University of Barcelona, Barcelona. SWEDEN: Huddinge Hospital, Huddinge. SWITZERLAND: Kantonsspital Basel, Basel; Kantonsspital Zurich, Zurich. TAIWAN: National Taiwan University Hospital, Taipei; Provincial Taoyuan General Hospital, Taoyuan. THE NETHERLANDS: Academisch Ziekenhuis, Leiden; Dr Daniël den Hoed Cancer Center, Rotterdam; University Hospital Leiden, Leiden; University of Nijmegen, Nijmegen. UNITED STATES: All Children’s Hospital, St. Petersburg, FL; Alta Bates Hospital, Berkeley, CA; Children’s Hospital, Cincinnati; City of Hope National Medical Center, Duarte, CA; Cleveland Clinic; Emory University School of Medicine, Atlanta; Hahnemann University, Philadelphia; Lackland Air Force Base, Texas; Loyola...
University Medical Center, Maywood; Latter Day Saints Hospital, Salt Lake City; Memorial Sloan-Kettering Cancer Center, New York; Medical College of Wisconsin, Milwaukee; Oklahoma Teaching Hospitals, Oklahoma City; Roswell Park Memorial Institute, Buffalo; Texas Children’s Hospital, Houston; UCLA-Center for Health Sciences, Los Angeles; University of Alabama, Birmingham; University of Florida, Gainesville; University of Kansas, Kansas City; University of Kentucky, Lexington; University of Minnesota, Minneapolis; University of Oklahoma, Oklahoma City; YUGOSLAVIA: Klinika za Unutrasnje Bolesti KBC-Rebro, Zagreb.

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GRAFT-VERSUS-HOST DISEASE IN THE EXPERIMENTAL ANIMAL

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Allogeneic bone marrow transplantation is the preferred treatment for patients with immunodeficiency disease and severe acute aplastic anaemia: it is currently also being compared to cytotoxic chemotherapy as maintenance therapy following first complete remission in leukaemic patients. Providing adequate numbers of stem cells are infused, and the patient has not been previously isoimmunised from exposure to donor antigens, engraftment is a limited problem. The greatest barrier to successful haematopoietic and immunologic reconstitution is graft-versus-host disease. A rabbit model has been developed in which this unique inflammatory process has been histologically defined, and is being used to examine immunologic manipulation with Corynebacterium Parvum and Cyclosporin A on survival. Using a specific strain combination of R female donors into NZW male recipients radiation control animals die aplastic with a mean of five days and autologous rescue results in haematopoietic reconstitution within 21 days. Allogeneic transplantation results in histologically proven graft-versus-host disease with wasting, diarrhoea and infection with 10% survival at 40 days, and no survival at 100 days. C. Parvum administration increases these figures to 58% and 30%, respectively. Administration of Cyclosporin A has 44% survival at 40 days and 33% survival at 100 days. These data establish activity for both C. Parvum and Cyclosporin A in the rabbit model, and their markedly differing action suggests that they may be used in combination in clinical transplantation.

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INTRODUCTION

In neither man nor experimental animals is it possible to consistently replace an irreversibly damaged organ or tissue without significant morbidity and mortality. Despite numerous unresolved problems, transplantation remains the most logical approach to managing these situations and, therefore, a major goal of physicians.

Many of the technical aspects of the procedures, although complex, have been perfected, but in the specific context of bone marrow transplantation two immunologic barriers exist, and these have markedly limited its clinical application. The first of these is graft rejection, which historically accounted for many of the early failures but differed in no important way from that encountered in renal, hepatic, and cardiac replacement. This problem has been approached by defining the number of marrow cells that need to be infused and, more importantly, by matching donor and recipient for antigens determined at the major histocompatibility complex. Isoimmunization from blood transfusion is a further mechanism that may compromise engraftment, but should be easily avoided by educating referring physicians.

Graft-versus-host disease (GVHD) is the second barrier to successful bone marrow transplantation. This unique phenomenon is a manifestation of immunologically competent cells being transferred to an immunocompromised recipient, where an inflammatory response is mounted against the antigen-bearing cells of the host. The organs predominantly affected are skin, gastrointestinal tract, and liver; but lymph nodes and lung may also suffer damage.

The morbidity and mortality from this syndrome are such that the potential of bone marrow transplantation remains far greater than its current clinical application. This is particularly unfortunate, since evidence is accumulating that it is the best form of treatment available for severe acute aplastic anemia (30) and combined immunodeficiency disease (6), while early results are encouraging in patients with myeloblastic (33) or lymphoblastic (34) leukemia transplanted in complete remission. For these reasons, much current research is aimed at trying to understand the pathogenesis of graft-versus-host disease and at systematically exploring agents that may be effective in either prophylaxis or management of this serious complication.

GRAFT-VERSUS-HOST DISEASE

In both man and in experimental animals the clinical syndrome is relatively easy to recognize. The
dominant features are cutaneous involvement, gastrointestinal symptoms, jaundice, and a decreasing performance status (13). Based on the severity and number of organs involved, four clinical stages are recognized. In Grade I, a maculopapular skin rash may be the solitary finding. In Grade II, the skin is more prominently involved and mild gastrointestinal symptoms with diarrhea, nausea, cramping abdominal pain, vomiting, and malabsorption occur. Liver involvement is reflected in jaundice, and elevated liver enzymes as well as plasma alkaline phosphatase. The clinical performance status is mildly impaired. In Grade III disease the symptoms are aggravated and clinical performance becomes markedly impaired. In Grade IV, the pattern and severity of signs and symptoms are similar but extreme constitutional disturbances are evident. Certain prerequisites are necessary, of which immunodeficiency in the recipient, infusion of immunocompetent lymphocytes from the donor, and antigenic disparity between donor and recipient are among the most important variables. Because of the uncertain way in which these three interact, attempts to control this immunologically mediated inflammatory phenomenon will vary widely.

There seems general agreement that the closeness with which donor and recipient are matched at the major histocompatibility complex correlates inversely with the severity of the graft-versus-host disease. Identity at the HLA-D locus appears to control the capacity of inflammatory cells to recognize differences existing at HLA-A and HLA-B loci. There is evidence for this in two children with severe combined immunodeficiency disease, who were transplanted with histoincompatibility at the latter two loci, but identity at the HLA-D locus. In both cases the reactions were less severe than had the A and B loci been major factors in the reaction (11,12). However, it is unlikely that this is the complete explanation, since identity at all sites presently identifiable on the major histocompatibility complex is still associated with GVHD in approximately 70% of patients (31,32). Furthermore, the development of the same syndrome in syngeneic transplants (26) suggests that other chromosomal regions may be important determinants of graft-versus-host disease.

More recently, evidence has been presented that GVHD may result from imbalance in immunoregulatory T-cell subsets, manifesting itself in the emergence of autotoxic T cells or autoantibodies (27). Thus, in patients with both acute and chronic GVHD, the TH{sub}s + T subset of cells, identified by specific heteroantiserum and containing the suppressor population, are reduced. The acute disease may be self-limiting and this could be explained by transient loss of the suppressor population following total body irradiation or cyclophosphamide conditioning. Abrogation of the GVHD could theoretically occur at a time when thymic function is reestablished and the suppressor TH{sub}s + T cell subpopulation is reestablished.

The same workers (27) point out that in chronic GVHD the cell aberration is more heterogeneous, with some of the T cells bearing Ia-like antigens. The latter only appear on the T cells once they have been activated, and in normal circumstances, are restricted to the B cells. In patients with chronic GVHD, one mechanism may be failure of the TH{sub}s + subset to regenerate, leaving helper cells (TH{sub}s - Ia+) unopposed. Alternatively, in vivo activation of the suppressor cells may overshoot with TH{sub}s +, Ia+ population being two or three times normal.
thereby suppressing the immune response. These studies do not elucidate the mechanisms whereby alloactivation or target-organ damage occurs, but they do provide evidence for regulatory imbalance in patients undergoing bone marrow transplantation and subsequently developing GVHD. This approach also provides an alternative classification for the syndrome.

In view of the uncertainty in the pathogenesis and the not inconceivable complications associated with therapy, attempts to predict the severity of GVHD are important, since this may provide a basis for modifying treatment. Since it is apparent that prevention is preferable to trying to reverse the established inflammatory process, a recent study demonstrating that pretransplantation assessment of natural killer cell activity directed against herpes simplex virus type 1 may have important prediction for the development of this disease is deserving of further evaluation (19).

At the present time immunosuppression, which is relatively nonspecific, remains the cornerstone of both prophylaxis and treatment. It is against this background that we have started evaluating cyclosporin A in both an experimental animal mode and in a clinical transplantation program.

**CYCLOSPORIN A**

Cyclosporin A (CS-A, OL 27 400) was discovered in early 1972 in the Research Laboratories of Sandoz Ltd in Basle. It is a hydrophobic cyclic peptide consisting of 11 amino acids with a molecular weight of 1203. The active compound is a fungal metabolite derived from cultures of *Cylindrocarpon lucidum* or *Trichoderma polysporum* (3).

In screening programs, cyclosporin A was found to have powerful immunosuppressive properties that differed in important and fundamental ways from the more conventional cytotoxic drugs and, of particular importance, have a remarkably low degree of myelotoxicity. Thus, while the target cell is clearly and specifically the lymphocyte in mice, rats, rabbits, dogs, and rhesus monkeys, the mechanism of action remains unknown.

Available evidence localizes the activity of cyclosporin A to the inductive or earliest phase arising in the immunocompetent cells as they respond to the mitogenic message. Cell division, by way of contrast, does not appear to be affected and, furthermore, no lymphocyte cytotoxic effects are demonstrable. The reduction in lymphoblast numbers, which may be striking, in vitro probably reflects prevention of stimulation rather than elimination of blast cells by cytolytic mechanisms (5). Expressed in another way, the primary effect of this agent is directed against the earliest cellular events in the lymphocyte that follow mitogenic stimulation and finds expression by preventing transformation into the blast cell.

The subpopulations of lymphocytes that may be affected have not been defined. However, while B cells are not directly suppressed, their production of antibody, together with that of plasma cells, is inhibited both in vitro and in vivo by the presence of CS-A implying that T-helper cells are the prime targets in its action.

The more critical study of T lymphocyte dysfunction is bedevilled by the many assay systems in use. As a result, controversy currently exists as to the role, if any, on the T-effector and T-suppressor subpopulations. Undoubtedly clarification of any selective action that cyclosporin A has on specific subsets of T lymphocytes is now a high research priority. It can, however, be said that it does not lead to selective clonal deletion but rather to the induction of a temporary suppressive state that is reversible when treatment is terminated (18,36).

Despite this persisting uncertainty, numerous laboratory correlates have been described in support of its interference with lymphocyte function (4,37) and powerful immunosuppressive activity has been reported in animal studies. For example, allograft survival has been prolonged after cardiac transplantation in rats (16) and pigs (8); and renal transplantation in rabbits (15), dogs (7), and in man (9). Cyclosporin A is capable of preventing or ameliorating GVHD in mice (3) and rats (35). Preliminary studies in man are also encouraging (25).

As with any new agent, side effects require special attention. These were originally defined using the administration of doses that far exceeded those needed to achieve effective immunosuppression. Here there was regression in thymus weight, cellular depletion of paracortical areas in lymph nodes or a white pulp in the spleen, and concomitant atrophy of lymphoid follicles (5). In the kidneys, there was swelling, vacuolization together with scattered necrosis, and regeneration of tubular cells.

In the liver, swelling and single hepatocyte necrosis was accompanied by centriflobular fatty degeneration. Of interest was mucosal angiopathy in the ileum (5). These findings characterized animals receiving doses associated with high mortality but reversed spontaneously within 4 weeks in animals who recovered (5).

In clinical studies some reversible nephrotoxic and hepatotoxic symptoms were found not necessarily associated with significant structural change when the tissues were examined histologically (9).

The toxicity of cyclosporin A is even more diffi-
cult to define in patients undergoing bone marrow transplantation where any side-effects need to be segregated from those that occur with high dose cyclophosphamide or lethal whole body irradiation. Nevertheless, there seems little doubt that varying degrees of renal dysfunction, hepatotoxicity, gastrointestinal tract symptoms, and bone marrow depression may be associated with cyclosporin administration (25). These can be reversed by careful adjustment to drug administration.

MATERIALS AND METHODS

Rabbits
New Zealand white (NZW) and R strain animals having an average weight of 2 kg were used throughout. The animals were individually housed and received water containing prophylactic sulpha-quinoxaline sodium (Embasin, May and Baker, Dagenham, England) and standard rabbit diet (Epol, Johannesburg), medicated with Amprolium (Merck and Company, Inc., USA). Temperature and weight were regularly charted. A full blood count and differential were done twice weekly. The biochemical profile was monitored regularly.

Radiation Technique
Animals were rendered aplastic by delivering 1200 rads total body irradiation from a central cobalt source placed 100 cm above the midplane of the prone but anesthetized animal. Radiation rates between 40 and 80 rads/min were shown not to differ significantly. In this model we have previously demonstrated (21) that uniform aplasia is produced without unacceptable radiation complications. Specifically, no gastrointestinal tract lesions were demonstrated. We did not find any difference between the technique and fractionalizing the radiotherapy, as suggested by others (22,23). In occasional animals, unexplained death occurred within 24 hr after administering the radiation and was ascribed to “radiation shock.” Current studies are evaluating the alternative radiation dose rate of 8 rads/min, and preliminary data indicate this regimen to be superior to the higher dose rates.

In a series of animals one or both of the femurs were shielded, and in another group autologous reconstitution was undertaken to provide controls to monitor both optimal rates of hematopoietic recovery and possible complications of the radiotherapy and transplantation procedure.

Allogeneic Bone Marrow Transplantation
Thirty-six hours after the lethal whole body irradiation the animals underwent allogeneic bone mar-
row transplantation. Between 2 and 4 × 10^6 nucleated cells per kilogram were transplanted from R females into NZW males. Standard mixed lymphocyte cultures (17) to demonstrate nonidentity at the major histocompatibility complex were not uniformly successful. Using a modified technique (20) antigenic disparity was demonstrated between the two strains and this was most striking when mitomycin treated lymphocytes derived from the mesenteric lymph node were used as antigen and appendiceal lymphocytes as responding or target cells. Results at day 3 gave stimulation indices between 1:3.6 and 1:13.9. In our experience even the latter technique does not produce absolutely uniform results, and we are currently examining modifications to enhance its sensitivity.

Cyclosporin A Administration
Rabbits were given 10 mg/kg daily of cyclosporin A by intramuscular injection for 28 days following transplantation. Plasma levels were monitored by high pressure liquid chromatography.

RESULTS

Radiation Controls
Irradiated animals developed increasing pancytopenia with platelet and granulocytic counts reaching their nadir between days 4 and 6, and all rabbits died. Mean survival (Fig 10.1) was 6.8 days (SE ± 0.08, range 2–11 days).

Autopsy studies at intervals showed rapidly increasing bone marrow hypoplasia, and the cause of death was established as infection in animals with widespread colonies of bacteria present in all organs. Minimal associated hemorrhage was present in the gastrointestinal tract of occasional rabbits. There was profound loss of lymphoid tissue throughout the gut, thymus, and spleen.

Femoral Shielding and Autograft Rescue
In animals having one or both femurs shielded, a reduction in white cell and platelet counts essentially similar to those of radiation controls were observed, but peripheral counts regenerated rapidly and were again normal by day 21 (Fig 10.2). The regenerating bone marrow, when studied serially, showed clonal regeneration of the previously aplastic marrow cavities. Histology was normal by day 30.

In the autografted animals who received between 2 and 4 × 10^6 nucleated cells per kilogram, peripheral blood values were indistinguishable from those observed with femoral shielding (Fig 10.2).

Anemia may be a problem and is aggravated by
Effect of Cyclosporin A on GVHD

**FIGURE 10.1.** Granulocytes and platelets follow similar patterns following 1200 rads midplane irradiation. The fall in circulating counts and the subsequent return to normal is not statistically different in animals having one or both femurs shielded during irradiation or following either allogeneic or autologous bone marrow transplantation. Absolute counts are plotted as a percentage of mean basal values against days that have elapsed following irradiation and transplantation. (-----) Femoral shielding; (---) allografts; (-----) autografts.

**FIGURE 10.2.** Observed survival in the four groups of rabbits. Median survival of irradiation controls was 6.8 days, allograft controls was 15 days, and allografted animals treated with cyclosporin was 40 days. At 100 days no allografted animal survived with cyclosporin A treatment in comparison to those who received this agent. In the latter group 33% of the original group were alive and well. In addition, cyclosporin A therapy reduced the incidence of histologically proven graft-versus-host disease from over 80% to below 25%.
blood sampling; microtechniques were therefore employed in these studies. In these animals, as opposed to radiation controls, the addition of prophylactic trimethoprim 10 mg, and sulphamethoxazole, 50 mg orally each day decreased deaths from infectious episodes and is routine practice in the animal transplantation program.

Allogeneic Bone Marrow Transplantation

In the allograft control experiments between 2 and 4 x 10^6 nucleated cells per kilogram resulted in granulocyte and platelet reconstitution in the peripheral blood, a pattern not statistically different from that demonstrated by animals undergoing radiation with femoral shielding, or after autografting (Fig 10.2). Similarly, the same pattern of bone marrow regeneration was found on serial histologic studies.

Since the study is designed to examine the effect of cyclosporin A on the incidence and severity of graft-versus-host disease, the rare animal dying before engraftment could be demonstrated was excluded from study.

The clinical findings in the allografted recipients were dramatically different from those in autograft controls. Gross weight loss was the striking feature, and at day 40 weights were, respectively, 0.8 and 2.8 kg. There was, in addition, patchy but typically extensive hair loss. Diarrhea was a variable feature, with cultures generally being negative.

Survival of animals (Fig 10.1) was 10% at day 40 and 0 at day 100. The cause of death was typically infection in the respiratory tract with nasal discharge of pus. Pneumonia was often present, with extensive destructive abscess formation being a feature. Histology of the skin, gastrointestinal tract, and liver were characteristic of graft-versus-host disease. In the skin aggressor lymphocytes were present in the epidermis, and spongiosis was prominent, particularly at the junction with the dermis. In the gastrointestinal tract loss of glands and patchy to total mucosal denudation were most striking in the small bowel. The liver showed infiltration of lymphocytes, loss of the limiting plate, and destruction of bile ducts by lymphocytes. Generalized lymphoid atrophy was evident in the spleen, lymph nodes, and thymus. These features were similar to those reported both in man (13) and in other experimental animals.

Cyclosporin Treated Allografted Animals

Two striking features were evident in these animals. First, they were uniformly in superior clinical condition with weights not significantly different from control animals. Hair distribution was normal, and diarrhea was not a feature. Second was the markedly different survival rate between allografted controls and rabbits receiving cyclosporin A after bone marrow transplantation (Fig 10.1). At day 40, 10% of the allograft controls were alive compared to 44% of those receiving cyclosporin A. This difference was even more obvious at 100 days when none of the controls survived, but 33% of those that had been treated were alive and well with normally functioning donor grafts.

Of particular note was the fact that the histologically recognizable features of graft-versus-host disease, which characterized the allografted control animals, were present in only 25% of animals receiving cyclosporin A. Furthermore, in the latter group death due to typical GVHD was unusual and was associated with incidental causes, such as perforated gastric ulcer and infection, of which bronchopneumonia and septicemia were the most frequent. It is presently not clear whether these terminal infections may be unusual expressions of GVHD modulated by cyclosporin A therapy.

Comments on Rabbit Study

Three points require comment. First, in the experimental animals the death rate appeared to accelerate after day 28 when cyclosporin A was discontinued. It therefore remains possible that longer periods of drug administration are necessary. These findings are entirely compatible with the suggestion that the action of this unique immunosuppressive agent is not lasting tolerance produced by selective clonal deletion, but is a transient phenomenon related to the presence of drug in the body (18,36). Furthermore, there are interesting parallels between this observation and those reported in a human transplantation study (24,25).

Secondly, it is noteworthy that in our animals, in contrast to experience with a similar model (29), we were unable to demonstrate any gross toxicity, and this is surprising. To some extent, the answer may be found in the third observation that high pressure liquid chromatography failed to demonstrate significant concentrations of cyclosporin A in the plasma of our animals. It therefore seems possible that the dosage administered may have been inadequate or the batch used had less biologic activity than we had anticipated. These questions are currently being reexamined. Nevertheless, the unequivocal and marked reduction in graft-versus-host disease and the striking prolongation in survival are further indicators that the plasma concentration of cyclosporin A may be less critical than currently accepted. Indeed, it remains to be proved that plasma levels are the most valid index of immunosuppressive activity. On the other hand these measurements are clearly the best presently available for
monitoring drug absorption and, in this way, anticipate toxicity.

CYCLOSPORIN TOXICITY IN CLINICAL STUDIES

Two reports from the Royal Marsden Hospital have indicated that encouraging results can be obtained from cyclosporin A administration (24, 25). Initially, early withdrawal of the drug led to exacerbation of GVHD and death of patients. Subsequently, there has been an appreciable reduction in the incidence of GVHD in patients receiving cyclosporin A, reversible side effects have been observed and, to date, no second malignant neoplasms have developed. The toxicity of the drug is an important concomitant of treatment and, in addition to experimental studies, we are able to add to this experience from some recently transplanted patients.

Case 1
A 21-year-old woman with severe acute aplastic anemia was referred for bone marrow transplantation. She required hemodialyses for a transient period of oliguric renal failure due to acute tubular necrosis (ATN) following sepsis. The infection was rapidly controlled with an antibiotic regimen of cephamandole, tobramycin, and metranidazole following replacement of her central venous catheter. In the diuretic phase of ATN she was given cyclophosphamide conditioning in preparation for her transplantation, and again became oliguric.

She received 2.8 x 10^6/kg nucleated cells from her compatible brother and 4 days later was pyrexial. A streptococcus was isolated from blood culture and after 6 days, the regimen was changed to the previous three-drug regimen, with temperature lysis within 48 hr. In this period, daily hemodialysis was used to maintain metabolic balance.

During this period cyclosporin A was given initially at 15 g/kg after each dialysis, and the dosage reduced to maintain a plasma level, measured by high pressure liquid chromatography, between 400 and 800 ng/ml. Engraftment was established by day 7, and no clinical evidence of graft-versus-host disease was present. Biochemistry showed a raised creatinine, which was controlled by regular hemodialysis; signs of low-grade cholestasis persisted. The patient died peacefully in her sleep on day 42, and autopsy revealed gastrointestinal tract hemorrhage as the probable cause of death. Renal tubules were regenerating. No histopathologic evidence of GVHD was present.

It is not certain as to the precise mechanism of her renal failure, although contributing factors include septicemia, nephrotoxic antibiotics such as tobramycin, and, for a short period of time, addi-

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The role, if any, played by cyclosporin A is likely to be small. Although this substance is nephrotoxic, this case establishes a precedent for its continued use in the anuric patient with the proviso that plasma levels are maintained by adjusting dosage and by efficient hemodialysis. It could be argued that the immunosuppression associated with renal failure may have been sufficient to avoid the need for cyclosporin A, but this point remains to be proved. There is, however, no doubt that the nephrotoxicity of this agent does not exclude its use in patients with renal insufficiency, as evidenced by histologically proved recovery in the presence of continued administration to this anuric individual.

Case 2
A 26-year-old male with acute lymphoblastic leukemia was transplanted in first complete remission; nucleated cells, 2.7 x 10^6/kg, from a compatible sister were given. Cyclosporin A was started on day 0, and for the first week given by intramuscular injection at a dose of 15 mg/kg; dosage was maintained at an average level of 10 mg/kg and plasma levels remained between 200 and 400 ng/ml.

Engraftment was established by day 10 and on post-transplant day 30, the patient developed a skin rash clinically compatible with the diagnosis of Grade 1 GVHD. Biopsy was, however, not diagnostic and suggested a drug-associated lesion. Simultaneously, liver enzymes and alkaline phosphatase started to rise rapidly and diarrhea developed. Despite careful maintenance of plasma cyclosporin levels, the patient developed marked anorexia, nausea, vomiting, abdominal pain and further elevations in liver enzymes and alkaline phosphatase.

In view of the equivocal skin biopsy and clear clinical deterioration, the possibility was considered that this entire syndrome may be drug related. Accordingly, cyclosporin A was reduced, and the patient started on oral prednisone, 1 mg/kg/24 hr. There was an immediate and dramatic clinical improvement, with a decrease in diarrhea. Within 10 days, liver enzymes and hepatic biochemical abnormalities had returned to normal. There has followed a gradual resolution of skin lesion. Cyclosporin was discontinued by accident on day 60; however, the patient is improving but still requires intravenous hyperalimentation for a severe wasting state. The cyclosporin has, therefore, not been restarted.

Although the explanation for the hepatotoxicity and gastrointestinal symptoms are uncertain, the rapid reversal of liver biochemical abnormalities support the relatively benign nature of these abnormalities. One possibility is that cyclosporin may have been directly implicated, but GVHD may have been responsible, despite the cyclosporin adminis-
tation, and the hepatic component reversed more rapidly than the gut lesion on corticosteroid administration. The residual severe wasting state may then reflect GVHD modified by both cyclosporin and subsequent steroid therapy. It is reassuring that serial absorption studies, including small bowel biopsy, show that gastrointestinal tract morphology and function returned to normal.

Thus, while these observations support the reversible nature of the hepatotoxicity in patients receiving cyclosporin A, they also draw attention to the fact that it is difficult to accurately attribute the changes solely to the drug. The converse also applies in that patients at risk of developing GVHD may express clinical and histologic features that differ from patients who are not receiving this agent.

Case 3

A 12-year-old male underwent bone marrow transplantation with $3 \times 10^9$/kg nucleated marrow cells from his compatible sister for severe acute aplastic anemia; he engrafted on day 9. Cyclosporin was given on the standard regimen and plasma levels monitored. On day 14 following transplantation, the patient developed conjugated hyperbilirubinemia, rises in alkaline phosphatase and liver enzymes, right upper quadrant abdominal tenderness, and an area of segmental ileus. Ultrasonography showed a large cystic mass considered to be possibly an intraabdominal abscess. At emergency laparotomy, a mucocele of the gallbladder due to edema of the common bile duct and cystic ducts was drained and the rest of the abdomen explored. No abnormality was demonstrated.

The liver and skin biopsy showed no evidence of graft-versus-host disease: the liver findings were compatible with drug-induced hepatotoxicity. Nephrotoxicity was never a problem.

On day 21 post-transplantation, a further small rise occurred in liver enzymes and cyclosporin A dosage was reduced to 10 mg/kg. The biochemical changes did not reverse and on day 25 the cyclosporin was withdrawn and corticosteroid therapy started. The liver function tests promptly started to improve and continued to do so steadily until his death from septicemia on day 34.

Autopsy showed marked edema, hyper trophy, and extensive ulceration of the bowel. No residual abnormality was present in the biliary system. Histologic examination, even in the absence of diarrhea, showed only minimal changes compatible with graft-versus-host disease.

The usual picture of GVHD was lacking from skin, liver, and gastrointestinal tract and this experience emphasizes the possibility that cyclosporin administration may modify both the clinical and histopathologic expression of GVHD. In this regard attention has already been directed to the way in which this agent can mask the typical cutaneous manifestation of GVHD(25). The rapidly reversible nature of the hepatotoxicity is again evident in this patient, even in the presence of transient obstruction to the biliary system.

COMMENT

Graft-versus-host disease remains the single greatest barrier to the more widespread use of bone marrow transplantation in clinical medicine. Attempts to diminish the severity of this clinical syndrome with prophylactic methotrexate or a variety of immunosuppressive regimens, ranging from antilymphocyte globulin to steroids and azathioprine (Imuran) have met with only limited success.

Preliminary experience with the unique immunosuppressive agent cyclosporin A has therefore justifiably initiated a series of studies in experimental animals and in man to define the place that the peptide might play in control of graft-versus-host disease.

In these experimental studies cyclosporin A was shown to be clearly of benefit in prolonging survival of allografted rabbits and that this was related to a prominent decrease in the incidence of histologically proved graft-versus-host disease.

It has not been possible to demonstrate toxicity of any type in the rabbit allograft model following cyclosporin A administration. This experience is in conflict with both our own experience in clinical transplantation and with that reported by other investigators using a similar rabbit model (29). It remains to be determined whether more detailed toxicity studies, correlated with varying plasma levels and differing schedules for administration in our rabbit model will reveal changes not evident in the earlier studies. This must take into account histology, ultrastructure, and functional measurements. In addition, evaluation of immunologic competence and correlation with an ability to handle infection await clarification.

Of particular note has been the absence of lymphoma or secondary malignancies in animals that received cyclosporin A and survived for prolonged periods of time. Here again, the question of duration and dosage of drug administration, period of observation after allografting, and associated conditioning regimens require careful analysis. These observations should be taken in the context of current studies in humans where early results are equally encouraging but toxicity is still not clearly defined.

In renal transplantation a word of warning has been sounded about the development of lymphoma (2,10). Nevertheless it should be recognized that
interstitial pneumonitis in 17, acute GVHD in 10, hemorrhage in seven, organ failure in six, adult respiratory distress syndrome (ARDS) in four, venoocclusive disease in three, chronic GVHD in three, and other causes in seven.

**DISCUSSION**

We have previously reported that allogeneic BMT in multiple myeloma produces a high complete remission rate, with a fraction of patients entering sustained long-term complete remission. Analysis of prognostic factors at that time demonstrated a significantly higher complete remission rate in patients with stage I disease at diagnosis, in patients who had received only one line of treatment, and in those who were in complete remission before conditioning for transplantation; however, although there were trends for longer survival in these patients, these were not statistically significant. In the present study, we were able to demonstrate significantly higher complete remission rates, and also significantly better survival in patients who were in stage I at diagnosis, who had received only one line of treatment before bone marrow transplantation, or who were in complete remission before conditioning as compared, respectively, with patients who were in later stages at diagnosis, had received two or more lines of treatment, or were not in complete remission before conditioning. In addition, we observed that females have a higher complete remission rate and better survival than males. In BMT for leukemia, the reason for a similar difference appears to be that results are poorer if female marrow is given to male recipients, than if female marrow is given to female recipients. A slight trend in this direction was seen, but it was not significant.

The age of the patient within the age bracket 23 to 59 years was not of significant importance for outcome. There was only a slight trend for patients less than 40 years to have better survival than older patients. This would agree with a recent study of patients with leukemia, which showed that the prognostic impact of age is small between 30 and 50 years.

The response rate was higher for both IgA and light-chain myeloma than for IgG myeloma. However, only IgA myeloma tended to have a better survival than IgG myeloma. Such a difference has generally not been noted with conventional chemotherapy, except in patients treated with combinations of interferon and melphalan. In one such study, patients with IgA or Bence-Jones—only myeloma had a significantly better response rate and survival than patients with IgG myeloma.

A limited number of patients were investigated for β2-microglobulin level at diagnosis. Patients with a high β2-microglobulin level (> 4 mg/L) tended to do worse than those with β2-microglobulin levels below this value. This was similar to the importance of β2-microglobulin in patients treated with conventional chemotherapy or ABMT.

The importance of procedural factors for outcome is difficult to investigate in this heterogeneously treated material. No superiority of any conditioning regimen to the conventional regimen of TBI plus cyclophosphamide was detectable. The follow-up time of patients treated with busulphan plus cyclophosphamide was shorter than for patients treated with TBI plus cyclophosphamide or other chemotherapeutic agents. Thus, it is difficult to make a fair comparison; however, the shape of the survival curve suggests that busulphan plus cyclophosphamide is not superior to any other treatment modality used. If anything, the tendency was for poorer survival, but it was not significant. Busulphan plus cyclophosphamide has recently been compared with TBI plus cyclophosphamide in a prospective, randomized study of other hematologic disorders by the Nordic Bone Marrow Transplant Group (NBMT). Although no significant difference in overall survival was found, patients with more advanced disease had a significantly poorer survival using busulphan plus cyclophosphamide than TBI plus cyclophosphamide, and it appeared that venoocclusive disease and hemorrhagic cystitis were more frequent in the busulphan-plus-cyclophosphamide group. The combination of busulphan plus cyclophosphamide for BMT in myeloma has been extensively evaluated by the Seattle group, who found that, in 12 of 15 assessable myeloma patients (N = 20), a complete remission was obtained, with complete remission defined as disappearance of the Ig assayed by immunofixation. The probability of survival at 3 years was 36%. As in the EBMT study, most patients had a relatively advanced stage of disease. Although this study was a phase I study that escalated the dosages of busulphan, it should be noted that four of 20 patients developed venoocclusive disease. This seems to support our view that venoocclusive disease may well be more common following busulphan plus cyclophosphamide as compared with TBI plus cyclophosphamide. Of 103 patients with myeloma who died in the present EBMT study, only three had venoocclusive disease determined as the primary cause of death.

Graft-versus-host prevention methods varied and it was therefore difficult to analyze any particular methodology. With T-cell depletion, acute GVHD was absent in 48% of patients. Without T-cell depletion, it was absent in only 32%. However, the lower frequency of GVHD with T-cell depletion did not translate into better survival or result in a higher relapse rate in comparison to those who were not T-cell-depleted. Thus, it was not possible to
many of these patients have had prolonged periods of renal disease and it is not certain that the development of lymphoma can be divorced with any degree of certainty from the previous periods when they may have been markedly immunocompromised from uremia. The failure to find secondary malignancies in animal models or in patients with severe acute aplastic anemia or leukemia undergoing bone marrow transplantation may therefore, in some way, be related to this variable as distinct from a complication of cyclosporin administration. This is another aspect of possible toxicity, presently the subject of evaluation in our animal experimental model.

In the specific context of bone marrow transplantation evidence is accumulating that graft-versus-host disease is capable of modification by cyclosporin A administration. The incidence of the syndrome is reduced but not abolished, and since the published data are relatively limited, many questions await answers. Foremost among these are the establishment of an optimum schedule on which the drug should be given, the minimum plasma level required to avoid toxicity and yet maintain an optimum degree of immunosuppression, the best tests to monitor the adequacy of the latter, the participation, if any, of metabolically active degradation products in the overall activity of cyclosporin therapy, and whether in vitro tests of immunologic incompetence correlate with in vivo effects of immunologic suppression in man.

What conclusion may be drawn from the available data?

Undoubtedly cyclosporin is both a unique and highly effective immunosuppressive agent. As such, it should continue to receive intensive study so that the mechanism of its action be defined. Already, sufficient reliable data are available to support further critical evaluation in selected clinical situations, and of these, transplantation programs have a high priority. It is appropriate that bone marrow grafting should be specially studied. Twin challenges of rejection and graft-versus-host disease must be overcome in order that its potential in an ever-expanding series of clinical indications may be realized. Finally, as with any new agent, early encouraging results and reasonable enthusiasm should be tempered by a responsible awareness of the possible side effects of cyclosporin administration. The interests of patients and scientists alike will be best served by the careful documentation and balanced reporting of benefits and limitations of treatment with what promises to be an exciting new form of treatment.

SUMMARY

Graft-versus-host disease (GVHD) is the most important cause of death in patients and animals following allogeneic bone marrow transplantation. Neither attempts to prevent the syndrome with methotrexate or treatment of the established disease with corticosteroids or antithymocyte globulin have significantly altered morbidity or mortality. The immunosuppressive agent cyclosporin A (CSA) is reported to be effective in therapy of GVHD in rodents. The present study was undertaken to extend this observation. Using a previously described animal model, New Zealand White strain of rabbits were irradiated with 1200 rads midplane to render them aplastic; this was followed by infusion of a bone marrow suspension obtained from R strain rabbit donors. The recipients were randomly allocated to receive either no treatment or 28 consecutive daily intramuscular injections of CSA (10 mg/kg). There was no evidence of renal, hepatic or hematopoietic toxicity associated with the CSA administration. Survival figures at 28 days in control animals were 1 of 8 (12.5%) and in those receiving CSA 11 of 18 (61%); at 100 days there were no survivors in the untreated animals and 6 of 18 (33%) of those receiving CSA were alive and well. Histology of tissue obtained at autopsy from the controls showed changes compatible with GVHD in all animals examined. In CSA treated animals that died before day 28, there was a lower incidence of these findings and death was due to infection. However, after 28 days GVHD was again manifest affecting predominately the gut. In none of our animals was there evidence of malignancy developing in the long-term survivors. It is concluded that, as in rodents, the administration of CSA to rabbits undergoing allogeneic bone marrow transplantation significantly prolonged survival.

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Improving the Outcome of Bone Marrow Transplantation by Using CD52 Monoclonal Antibodies to Prevent Graft-Versus-Host Disease and Graft Rejection

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Graft-versus-host disease (GVHD) is a major cause of mortality and morbidity after allogeneic bone marrow transplantation, but can be avoided by removing T lymphocytes from the donor bone marrow. However, T-cell depletion increases the risk of graft rejection. This study examined the use of CD52 monoclonal antibodies to eliminate T cells from both donor marrow and recipient to prevent both GVHD and rejection. Seventy patients receiving HLA-identical sibling transplants for acute mylogenous leukemia (AML) in first remission were studied. An IgM (CAMPATH-1M) was used for in vitro depletion of the graft and an IgG (CAMPATH-1G) for in vivo depletion of the recipient before graft infusion. No posttransplant immunosuppression was given. Results were compared with two control groups: (1) 30 patients who received bone marrow depleted with CAMPATH-1M, but no CAMPATH-1G in vivo; and (2) 49 patients reported to the International Bone Marrow Transplant Registry (IBMTR) who received nondepleted grafts and conventional GVHD prophylaxis with cyclosporin A (CyA) and methotrexate (MTX). The incidence of acute GVHD was 4% in the treatment group compared with 39% in the CyA/MTX group (P < .001).

HIGH-DOSE CHEMOTHERAPY and radiotherapy followed by transplantation of hematopoietic stem cells can cure patients with leukemia. However, allografts have several adverse effects, the most serious of which is graft-versus-host disease (GVHD). It can result in severe damage to skin, liver, and gut, frequently leading to death or chronic disability. To control GVHD, immunosuppressive drugs such as cyclosporin A (CyA), methotrexate (MTX), and corticosteroids are administered posttransplant. However, even with combined cyclosporin and methotrexate, GVHD remains the single most common cause of death after allogeneic transplants.

Chronic GVHD was also exceptionally low in the treatment group (8% vs 36%; P < .001). The problem of graft rejection, which had been frequent in the historic CAMPATH-1M group (31%), was largely overcome in the treatment group (8%). Then, transplant-related mortality of the treatment group (15% at 5 years) was lower than for the CyA/MTX group (26%; P = .04). There was little difference in the risk of leukemia relapse between the treatment group (30% at 5 years) and the CyA/MTX group (29%). Survival of the treatment group at 6 months was better than the CyA/MTX group (82% vs 78%), although at 5 years the difference was not significant (62% vs 58%) and neither was the difference in leukemia-free survival (69% vs 55%). We conclude that T-cell depletion is a useful strategy to prevent GVHD, provided that measures are taken to ensure engrafment. Using CAMPATH-1G to deplete residual host lymphocytes is a simple and practical method to do this. At least in AML, the beneficial reduction in GVHD can be achieved without an increased risk of relapse.

For many years it has been known that GVHD can be prevented by depleting T lymphocytes from the donor bone marrow and a variety of methods were developed to accomplish this. One of the most widely used has been the monoclonal antibody (MoAb) CAMPATH-1M, a rat IgM antibody that recognizes the CD52 antigen. CD52 is abundantly expressed on all human lymphocytes and is an exceptionally good target for cell lysis by antibody with human complement; this provided a simple method for purging the donor T cells. Prior studies demonstrated the efficacy of T-cell depletion and consequent reduction in GVHD. However the benefit was

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offset by an increased risk of graft rejection by residual host T cells,11,12 and some patients suffered an increased risk of leukemia relapse due to the loss of graft-versus-leukemia effects contributed by donor lymphocytes.13,17 Animal models18,19 and clinical experience20 showed that graft rejection might be overcome by increasing the immunosuppression of the recipient before transplantation. One way of delivering this, without adding to the toxicity of the conditioning regimen, is to use MoAbs to deplete residual host T cells.21 A rat IgG2b CD52 antibody, CAMPATH-1G, effectively depletes human lymphocytes in vivo.22 Like CAMPATH-1M, it can activate human complement, although this is not sufficient for systemic T-cell depletion. Rat IgG2b also binds human Fc receptors and engages cellular killing mechanisms.23

A combined strategy using CAMPATH-1M to T-cell-deplete donor bone marrow and intravenous CAMPATH-1G to ablate residual host immunity has been used in more than 600 transplants worldwide, primarily in transplants from unrelated donors.11,24,25 We report here results of 70 HLA-identical sibling transplants for acute myelogenous leukemia (AML). We compare the results here to a historical first group receiving CAMPATH-1M-treated marrow but no intravenous CAMPATH-1G and second to a matched group of concurrently treated patients reported to the International Bone Marrow Transplant Registry (IBMTR) who received unmanipulated transplants and posttransplant cyclosporin plus methotrexate for GVHD prophylaxis.

MATERIALS AND METHODS

MoAbs. CAMPATH-1G26 was prepared from the culture supernatant of hybrid myeloma cell culture in a hollow-fiber fermentor (Alice, Minneapolis, MN). It was purified by affinity chromatography on protein A sepharose, followed by ion exchange chromatography on S-Sepharose, and formulated in phosphate-buffered saline. CAMPATH-1M27 was prepared from hybrid myeloma cells using three methods: (1) acute fluid fractionated with ammonium sulphate, (2) hollow-fiber culture supernatant fractionated with ammonium sulphate, and (3) culture supernatant from stimulated ferments purified by affinity chromatography on protein A sepharose (this was performed by Wellcome Biotech [Beckenham, UK], who provided some of the antibody for this study). Batches prepared by each method were tested in a variety of analytical systems. All had comparable potency for complement-mediated cell lysis and insignificant effect on colony-forming cells.22,29 Process (1) was used for the historic controls, process (2) was used for the study patients at London and Riyadh, and process (3) was used for the study patients at Ulm.

In vitro T-cell depletion of bone marrow. A similar depletion procedure was used for all transplants, as described previously.11,12 Donor bone marrow was harvested in the usual way and processed using a cell separator to prepare a cell concentrate in balanced salt solution (containing Ca21) that was free from plasma and depleted of red blood cells and granulocytes. The volume of the mononuclear cell suspension was adjusted so that the cell density did not exceed 5 × 106/ml, and CAMPATH-1M was added to give a final concentration of 0.1 mg/ml. The mixture was incubated for 10 to 20 minutes at room temperature, and then donor serum was added to a final concentration of 25% (vol/vol). It was then incubated for a further 20 to 45 minutes at 37°C. The treated bone marrow was washed once before infusion. Experiments just shown that differences in antibody batch or incubation timing did not materially affect the efficacy of T-cell depletion (G.H., unpublished work). The fraction of residual T cells was measured using standard methods, according to the practice in each center, either by F-rosettes or by fluorescence-activated cell sorting (FACS) analysis using appropriate T-cell-specific mouse MoAbs. (All of the study patients were assessed by FACS analysis using CD3 antibodies to enumerate T-cells.)

In vivo administration of CAMPATH-1G. Patients were treated with 20 mg/kg of CAMPATH-1G over a period of 5 days at the beginning of the pretransplant conditioning therapy. Each dose was diluted in 250 ml of normal saline and infused intravenously over 3 hours. To minimize the expected systemic first dose reaction, most patients received medication before the first antibody infusion, either 0.5 to 1 g prednisolone (Ulm) or 100 mg hydrocortisone plus 50 mg diphenhydramine (Royal Free, Riyadh), followed by 3× 1 g as needed daily.

Patients: Study group. Three transplant centers participated in the study: Ulm University Hospital (Ulm, Germany), Royal Free Hospital (London, UK), and King Faisal Hospital (Riyadh, Saudi Arabia). The original plan was to include all patients over 13 years with acute leukemia in first remission transplanted from HLA-matched siblings with the intent of comparing results in acute lymphoblastic leukemia (ALL) and AML. Because very few patients with ALL were recruited, this analysis focuses on patients with AML. (Inclusion of the ALL patients would not significantly affect the results.) The conditioning regimen was determined according to the standard protocol of each center. All included cyclophosphamide plus total body irradiation (TBI), which was administered as a single dose in 23% patients (at the Royal Free) and multiple fractions in the others (at Ulm and Riyadh). Additional chemotherapy (busulphan) was administered in 6% of patients (Royal Free only). None of the patients received any additional lymphoid irradiation and none received posttransplant immunosuppression. There was no selection of patients according to cytogenetic risk group and data on risk groups were not reported systematically. Each center recruited consecutive patients provided that they gave informed consent. The numbers of patients and conditioning regimens at each center are shown in Table 1.

Patients: Historic CAMPATH-1M controls. A database is maintained by Gh of all transplants using CAMPATH-I antibodies. It contains information on the patient and donor characteristics and transplant outcomes. It is in a condition of antibody supply that are regularly reported to the CAMPATH users database. Controls were selected using the following criteria: (1) patients more than 13 years of age, (2) transplants for AML in first remission, (3) HLA-identical sibling donors, (4) conditioning regimens based on cyclophosphamide and TBI, (5) no lymphoid irradiation, (6) no intravenous CAMPATH-1G or other antibody, (7) T-cell depletion with CAMPATH-1M, and (8) no posttransplant immunosuppression. Fifty patients meeting these criteria were identified; 26 were transplanted at the same three centers as the 70 study patients.

Patients: IBMTR controls. A second control group of patients receiving non-T-cell-depleted transplants between 1984 and 1995 was selected from the IBMTR database using the same criteria described above, except that patients from the three study centers were excluded and GVHD prophylaxis was with combined CyA and MTX.28 IBMTR is a voluntary study group of over 390 transplant centers worldwide that contribute detailed data to a Statistical Center at the Medical College of Wisconsin. Participants are required to report all consecutive transplants.

Table 1. Conditioning Regimens Used at the Three Study Centers

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Ulm</th>
<th>Royal Free</th>
<th>Riyadh</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>41</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>TBI fractions (no.)</td>
<td>6</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>TBI total dose (rad)</td>
<td>1,200</td>
<td>750</td>
<td>1,200</td>
</tr>
<tr>
<td>TBI dose rate (rad/min)</td>
<td>12</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Time between last dose of antibody and transplant</td>
<td>5 d</td>
<td>1-3 d</td>
<td>5 d</td>
</tr>
<tr>
<td>Additional chemotheraphy (no. of patients)</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
plants. The IBMTR database includes 40% to 45% of allogeneic transplant recipients since 1970. Computerized error checks, physician review of submitted data, and on-site audits of participating centers ensure data quality. Selection of the control group proceeded as follows: patients with AMI, transplanted in CR1 from 1964 through 1995 (inclusive), N = 2,940; exclude the three study centers, N = 2,759; select patients transplanted from HLA-identical siblings, N = 2,429; select cyclophosphamide + TBI with no ATG for conditioning, N = 1,307; select MTX plus CyA for GVHD prophylaxis, N = 512; and select patients more than 13 years of age at transplant, N = 459.

These 459 cases were reported to the IBMTR by 93 transplant centers.

Statistical analysis. Characteristics of the treatment groups were compared using the \( \chi^2 \) test for categorical variables and the Wilcoxon two-sample test for continuous variables. Comparing outcomes between the treatment groups required adjustment for the differences in baseline characteristics and prior therapy. First, associations between outcomes and potential prognostic variables were evaluated in each group separately using Cox proportional hazards regression with a forward stepwise approach.21 The outcomes considered were rate of engraftment (days to reach 0.3 \( \times 10^9 \) neutrophils/L), graft failure, transplant-related mortality (TRM; defined as death in continuous complete remission), relapse, survival, and leukemia-free survival. Variables considered were age at transplant, recipient gender, donor gender, and year of transplant. Variables significantly associated with the outcome in any treatment group were included as covariates in subsequent comparisons. Possible interactions between significant covariates and the type of treatment were tested. The proportionality assumption of the Cox model was tested by adding a time-dependent covariate for each covariate.22 The proportionality assumption did not hold for treatment effects on survival, indicating that the relationship between treatment and survival outcomes differed over time. To determine regions of the treatment period where the relative risk of mortality between different treatments was a constant, a series of Cox models with different cut-off time points for time-dependent treatment effects were fitted.23 The final model chosen was the one giving the largest partial likelihood. In this model, treatment was considered as a time-dependent covariate with different coefficients from 0 to 6 and greater than 6 months since transplantation. The adjusted relative risks (95% confidence intervals and \( P \) values) of the study group versus historic group and study group versus MTX/CyA group were calculated for each outcome. Where appropriate, confidence intervals were calculated based on a log transformation.

RESULTS

T-cell depletion in vivo by CAMPATH-1H. Several methodologies were used in the years to estimate the fraction of residual T-cells in treated marrow (Table 2). The measured fraction of residual T-cells in the study group (median, 0.4%) was lower than in the historic control group (median, 1.0%; \( P = .024 \)), but the result must be treated with caution owing to the potential variability in the measurement of small numbers of cells. The total number of mononuclear cells infused varied significantly between each of the three study centers and between the study group (median, 1.0 \( \times 10^9 \) /kg) and the historic control group (median, 2.15 \( \times 10^9 \) /kg; \( P < .001 \)). Consequently, the calculated numbers of T-cells infused differed significantly between the study group (median, 0.2 \( \times 10^9 \) /kg) and the historic group (median, 1.7 \( \times 10^9 \) /kg; \( P < .001 \)).

Lymphocyte depletion in vivo by CAMPATH-1G. CAMPATH-1G treatment was started before other components of the conditioning regimen, so that antibody effects could be determined. All patients had rapid and profound depletion of blood lymphocytes. The number of clonable T-cells that could be recovered from recipient blood samples obtained 1 to 2 days after the CAMPATH-1G infusions was reduced by 2.5 to 3 logs compared with pretreatment samples.23 In one study with CAMPATH-1G or CAMPATH-1H, there was generally a first-dose effect of fever, often with rigors and nausea, which is related to a release of cytokines.24,25 In two patients the reaction was severe, and it was decided to discontinue CAMPATH-1G. (These patients are still included in the analysis.) All other patients had diminished reactions to the second and subsequent doses.

Effect of year of transplant. To determine whether patients transplanted before 1990 could be included in comparative analyses, we examined the IBMTR dataset for differences between patients transplanted in 1984 through 1989 compared with 1990 through 1995. There were no statistically significant differences in the actuarial risks of transplant-related mortality (26% vs 23% at 4 years), relapse (26% vs 33% at 5 years), survival (57% vs 58% at 5 years), or leukemia-free survival (54% vs 49% at 5 years). Therefore, all data from 1984 through 1995 were pooled for subsequent analyses.

Comparison of prognostic features of the study and control groups. Characteristics of the study and control groups are shown in Table 3. The most significant difference was in age. The median age of the study group (36 years) was significantly higher than the historic control group (30 years; \( P = .003 \)) or the CyA/MTX group (31 years; \( P = .03 \)). There was also a difference in the gender of patients between the study group (57% male) and the historic control (58% male; \( P = .04 \)), but not the CyA/MTX group (50% male). The potentially confounding effect of patient age on the outcome has been adjusted in a multivariate model (see below).

Comparison of outcome for study and control groups. Univariate analyses of outcome are shown in Table 4. Engraftment was significantly slower in both CAMPATH groups compared with the CyA/MTX group (Fig 1). Even disregarding patients who did not engraft at all, there was a delay of 1 day in

<table>
<thead>
<tr>
<th>No. of T-cells infused</th>
<th>14 (0.1-1.4)</th>
<th>0.5 (0.2-0.8)</th>
<th>0.1 (0.1-2.4)</th>
<th>1.0 (0.1-4.0)</th>
<th>2.0 (0.1-6.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cells (%)</td>
<td>0.8 (0.6-1.3)</td>
<td>0.2 (0.1-2.7)</td>
<td>0.2 (0.1-5.0)</td>
<td>0.4 (0.1-7.3)</td>
<td>1.9 (0.5-10.0)</td>
</tr>
<tr>
<td>T-cells infused (10^9/kg)</td>
<td>0.0 (0.0-5.4)</td>
<td>0.12 (0.0-1.2)</td>
<td>0.04 (0.0-2.2)</td>
<td>0.2 (0.0-5.4)</td>
<td>1.7 (0.2-25.0)</td>
</tr>
</tbody>
</table>

Cell numbers are reported as medians, with the ranges in parentheses. There was a statistically significant difference between each study center in the total numbers of mononuclear cells (\( P < .001 \)), but there was no significant difference in the percentage of residual T-cells or the total numbers of T-cells infused. However, there was a significant difference between the study group and the historic control group with regard to each of these parameters (\( P = .024 \) for percentage of T cells and \( P < .001 \) for total T cells).
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Table 3. Features of Study and Control Groups

<table>
<thead>
<tr>
<th>Study (n=2)</th>
<th>Historic (n=1)</th>
<th>CyA/MTX (n=3)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>70</td>
<td>50</td>
<td>459</td>
</tr>
<tr>
<td>Median year of transplant</td>
<td>92 (87-96)</td>
<td>86 (84-94)</td>
<td>50 (84-95)</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>44</td>
<td>116</td>
<td>58</td>
</tr>
<tr>
<td>Male patient</td>
<td>57%</td>
<td>38%</td>
<td>50%</td>
</tr>
<tr>
<td>Male donor</td>
<td>61%</td>
<td>52%</td>
<td>54%</td>
</tr>
<tr>
<td>Median age at transplant</td>
<td>56 (14-50)</td>
<td>30 (14-47)</td>
<td>31 (14-56)</td>
</tr>
</tbody>
</table>

The extreme range of the data is shown in parentheses. Probabilities (P) are calculated by the χ² test or Wilcoxon two-sample test as appropriate.

the median time to reach 0.5 × 10⁹ neutrophils/L for the historic CAMPATH group and 5 days for the study group. There was a significant variation in time to engraftment among the three study centers. The median time to reach 0.5 × 10⁹ neutrophils/L at UAB was 23 days, whereas at the Royal Free and Riyadh, the median was 30 days (P = .001). Platelet engraftment was not reported in this study.

The study group had a significantly lower risk of rejection than the historic group (65% vs 51%; P = .0003) but a higher risk than the CyA/MTX group (26%, P = .03; Fig 2). The incidence of both acute and chronic GVHD was significantly lower in the study group compared with either of the two control groups. Only 3 of the 70 study patients developed grade 2 acute GVHD and 2 developed mild/moderate chronic GVHD; there were no more severe cases.

Table 4. Outcome According to Treatment Group

<table>
<thead>
<tr>
<th>% Probability (95% confidence interval)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study (n=2)</td>
<td>Historic (n=1)</td>
</tr>
<tr>
<td>0.5 × 10⁹ neutrophils/L</td>
<td></td>
</tr>
<tr>
<td>By day 21</td>
<td>23 (14, 34)</td>
</tr>
<tr>
<td>By day 30</td>
<td>78 (67, 87)</td>
</tr>
<tr>
<td>By day 60</td>
<td>94 (87, 98)</td>
</tr>
<tr>
<td>Grade I/II</td>
<td></td>
</tr>
<tr>
<td>At 1 mo</td>
<td>4 (1, 11)</td>
</tr>
<tr>
<td>At 12 mo</td>
<td>6 (2, 13)</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td></td>
</tr>
<tr>
<td>Grade I/IV</td>
<td></td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td></td>
</tr>
<tr>
<td>Grade II/IV (limited-extensive)</td>
<td>3</td>
</tr>
<tr>
<td>Transplant-related mortality</td>
<td></td>
</tr>
<tr>
<td>At 3 yrs</td>
<td>15 (7, 25)</td>
</tr>
<tr>
<td>At 5 yrs</td>
<td>15 (7, 25)</td>
</tr>
<tr>
<td>Relapse</td>
<td></td>
</tr>
<tr>
<td>At 3 yrs</td>
<td>27 (17, 39)</td>
</tr>
<tr>
<td>At 5 yrs</td>
<td>30 (18, 42)</td>
</tr>
<tr>
<td>Survival</td>
<td></td>
</tr>
<tr>
<td>At 3 yrs</td>
<td>65 (53, 76)</td>
</tr>
<tr>
<td>At 5 yrs</td>
<td>62 (50, 73)</td>
</tr>
</tbody>
</table>

The outcome is reported, together with the 95% confidence interval. Significance (P) values in this table (except for GVHD) were calculated from a univariate log-rank test (ie, they do not take into account any potential covariates).
group (1%). It was not possible to determine the cause of graft failure in every case, but it is likely that most, if not all, were due to immunological rejection. Therefore, both early and late graft failure were analyzed together. Patients were treated by reinfusion of stored autologous marrow (5 historic control and 1 study) or by a second transplant of un fractionated allogeneic marrow from the original donor (6 historic control and 3 study). In 11 cases, these rescue procedures resulted in successful engraftment, but many of the patients suffered further complications and eventually 12 of 15 patients died in the historic group (9 from graft failure) and 4 of 4 patients died in the study group (all from relapse).

Multivariate analysis. The results were further analyzed using the Cox proportional regression model to test for interactions with prognostic factors that might have affected the results of univariate analyses (Table 5). Variables used in building the model were age, year of transplant, patient gender, donor gender, and treatment group. Outcomes were rate of engraftment, graft failure, transplant-related mortality, relapse, survival, and leukemia-free survival. There was a significant association between age and transplant-related mortality. The relative risk of transplant-related mortality for patients more than 30 years of age was 1.53 times that for patients under 30 years (95% confidence interval, 1.08 to 2.15; \( P = .02 \)). However, the relationship between patient groups and outcome was similar for young and old patients. In the final model, allowing for the effect of age, there was less than half the risk of transplant-related mortality in the study group compared with the CyA/MTX control (relative risk, 0.45; \( P = .02 \)). Tests for proportionality indicated that the effect of the study treatment on survival differed at different times after the transplant. In the first 6 months after the transplant, survival of patients in the study group was significantly higher than the control group; among patients surviving 6 months, subsequent survival was similar in the study group versus the two control groups. It is not surprising that no significant difference in long-term survival could be shown, because the size of the study did not give it sufficient power to demonstrate even a 10% difference.

Causes of death. The underlying causes of death in each patient group are reported in Table 6. Because of the small numbers in the study and historic control group and the difficulties inherent in assigning a single cause of death to some patients, we did not attempt formal statistical analysis. The most frequent cause of death in the study group was relapse (15 patients), followed by infection (7 patients). There were 3
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Fig 6. Probability of leukemia-free survival.

Deaths from infection in the study group after 6 months: 1 varicella zoster + aspergillosis (day 191), 1 varicella zoster + hepatitis C (day 858), and 1 unknown organism (day 387).

DISCUSSION

Since the early clinical trials of T-cell depletion, it was realized that graft rejection by residual host T cells was a major problem that negated much of the clinical benefit of avoiding GVHD. Graft rejection could be reduced, but not eliminated, by giving extra whole body γ-irradiation or by administering posttransplant cyclosporine. Animal experiments showed that anti-T-cell MoAbs could be used to escalate the immunosuppression without toxicity, and this prompted the collaborative CAMPATH users group to begin a number of pilot studies using CAMPATH-1G to deplete residual host T cells. These studies gave encouraging results, but it was possible that other improvements in transplant protocols over time might have influenced the outcome. Therefore, we performed the present comparison with a large contemporaneous control group of patients selected from the IBMTT database. A second problem associated with T-cell depletion is the loss of beneficial graft-versus-leukemia effects. This is well documented for patients with chronic myeloid leukemia (CML), but it is not clear whether there is a significant effect in patients with acute leukemia. In this analysis, we evaluated relapse in patients transplanted for AML in first complete remission.

This study convincingly demonstrates the effectiveness of the combined CD52 antibodies in dramatically reducing the risk of acute and chronic GVHD, without posttransplant immunosuppression. There were no cases of severe acute or chronic GVHD in the study group. Similar results have been reported when the same antibody protocol was used in other transplant indications. The addition of CAMPATH-1G in vivo posttransplant resulted in lower GVHD rates than those seen in the historic controls, which might be due to additional depletion of donor T cells in vivo by residual CAMPATH-1G at the time of transplant. The measured extent of T-cell depletion by CAMPATH-1G was greater for the study patients than the historic controls, but the significance of this is hard to assess due to the technical difficulties in accurate measurement of small numbers of T cells and the fact that actual depletion is likely to have been greater than measured, because residual T cells would be coated with CAMPATH-1G antibody and lysed when they encounter fresh complement after infusing of the bone marrow.

Importantly, the study group also had a significantly lower risk of graft failure than the historic control (6% at 12 months). This is still higher than in the non-T-cell-depleted group (2%), but acceptable, given the much lower risk of GVHD. Possibly graft failure would be reduced still further if larger numbers of stem cells were infused, as is now possible using peripheral blood harvests. Despite the improvement in overall engraftment, the speed of engraftment, as measured by the time to reach 0.5 × 10^10 neutrophils/L, was significantly slower in the study group compared with either control group (after excluding graft failure). The most likely explanation for this delay is the comparatively small number of mononuclear cells infused in the study group, especially because the greatest delay was observed at the two centers where the smaller numbers of cells were infused (Table 2). Experimental models have shown that speed of engraftment is related to total stem cell dose, and this has been confirmed in a recent multivariate analysis of the whole CAMPATH users database (G.H. and S.P. Cobbold).
unpublished work). Rapid engraftment has been reported with CAMPATH-1–treated stem cells from peripheral blood. The relative risk of transplant-related mortality was significantly lower in the study group compared with the CyA/MTX group (15% vs 26%). This is most likely due to reducing the incidence and severity of GVHD, although we cannot rule out a benefit due to the avoidance of toxicity of the immunosuppressive drugs themselves or consequent infection risks. One of the most important parameters in the long-term follow-up was the risk of relapse, because it has been shown that T-cell depletion increases relapse risk substantially for CML, and it is suggested that there could be a modest increase in risk for acute leukemias. In contrast, we found no significant difference between the study group (30% risk of relapse at 5 years) and the CyA/MTX group (29%), supporting the concept that the impact of T-cell–mediated graft-versus-leukemia reactions is minimal in patients transplanted for acute leukemia in first remission. Overall survival was significantly better in the study group compared with the CyA/MTX group up to 6 months; subsequent survival and leukemia-free survival were slightly, but not significantly better.

Prospective randomized studies are often thought to be the gold standard in evaluating new treatments, but their application in transplantation is hindered by the relative infrequency of the diseases treated and the fact that only a minority of patients have suitable donors. This makes accrual of sufficient patients difficult, if not impossible. We were primarily interested in measuring differences in the risk of graft failure, where the expected results were in the range of 2% to 15%. Hundreds of patients would be required to give an adequate power. Fortunately, a better alternative is available. Large clinical databases, such as the one maintained by the IBMTR, contain data on a large proportion of transplant recipients worldwide with details of prognostic and treatment factors that allow application of sophisticated statistical techniques to adjust for differences between groups and exploit the power of large numbers. In the current study, we identified 459 suitable controls receiving the most common approach to GVHD prophylaxis against which the combined antibody strategy could be compared. Unlike many prospective randomized trials, in which significant differences are sometimes attributed to unusually poor performance of the control group, we can be sure that our CyA/MTX control group is truly representative. The accuracy of the control data are confirmed by published results from the European Transplant Registry on an overlapping set of patients, where the outcomes are superimposable. However, it might be argued that the three study centers shared some favorable factor in common other than the treatment protocol. In fact, the three centers were more remarkable for their diversity in approaches to transplantation. Furthermore, this idea is negated by the published comparison from one center (Ulm) of study patients compared with their own CyA/MTX control group. The results are very similar to those presented here, except for the smaller numbers of control patients. The limitations of our analysis should be recognized, particularly the difficulty of allowing for possible differences in relapse risk according to AML subtype, prior therapy, and conditioning regimen, but we can be confident that the control group is representative of contemporary clinical practice.

Immunoreconstitution was not specifically studied here, but results for marrow transplants depleted with CAMPATH-1M have been reported previously. All of these reports agree that lymphocyte recovery, particularly of CD4+ cells, is slow compared with T-replete transplants. There does not appear to be a substantial long-term mortality as a result of opportunistic infections, but this requires continued surveillance. Some groups report an early increase in the frequency of cytomegalovirus (CMV) reactivation, although this did not necessarily lead to severe clinical disease. The cellular distribution of the CD52 antigen may be fortuitous in this regard. NK cells are relatively spared by CAMPATH-1, and it has been suggested that they may play a role in control of CMV disease and have an antileukemia effect. However, B cells are efficiently depleted. Elsewhere, we have reported that T-cell depletion with CAMPATH-1 antibodies does not give rise to an excess of B-cell lymphoproliferative disorder, in contrast to current methods of T-cell depletion. We believe this is because depletion of donor B cells removes both a potential reservoir of virus and its major target.

In this trial, CAMPATH-1G was used as a form of monoclonal antilymphocyte globulin to achieve the additional depletion of recipient lymphocytes required to permit engraftment of T-cell–depleted bone marrow. The positive outcome is in accord with animal experiments, confirming that graft failure was caused by lymphocyte-mediated rejection. The logical development is to use the IgG antibody CAMPATH-1G (or its humanized equivalent CAMPATH-1H) for depletion of both donor and recipient T cells, and current trials are aimed at developing the simplest and most effective way to administer it—either in vivo before and after the transplant or as a single dose, mixed and infused with the donor bone marrow. This study confirms that T-cell depletion is the best way to prevent GVHD. Our approach largely avoids graft rejection and does not result in an increased risk of relapse, at least in AML patients. The disease-free survival achieved is at least as good as with conventional regimens, but with the following important advantages: (1) posttransplant immunosuppression is no longer needed and (2) almost complete elimination of acute and chronic GVHD should translate into a substantially better quality of life for the survivors.

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The authors are indebted to many colleagues who played an important part in the production of antibodies, care of patients, data collection, and analysis, including Jenny Phillips and the staff of the Therapeutic Antibody Centre (Cambridge, UK), Marcus Wieseth, Bernd Herzenstein, Renate Arnold (Ulm, Germany), Mike Hancock, Ian MacDonald, Grzyna Galazwicz, Chris Collins (Royal Free, London, UK), Hugh Culkin, Andrew Paddison, Peter Ernst, and Kirill Shafik (Royal, Saudi Arabia) as well as the following transplant teams throughout the world who contributed to the registry data: Australia: Royal Prince Alfred Hospital, Camperdown; Alfred Hospital, Prahran; Westmead Hospital, Westmead; Austria: Univ. Klinik für Innere Medizin I, Vienna; Belgium: A.Z. Sint-Jan, Brugge; Cliniques Universitaires Saint-Luc, Brussels; University Hospital Gasthuisberg, Leuven; University of Liege, Liege; Brazil: Hospital de Clinicas, Curitiba; Centro Nacional de Transplante de Medicina Osasco-CEMO, Rio de Janeiro; Canada: Ontario Cancer Institute, Princess Margaret Hospital, Toronto; China: Bel Tai Ping Lu Hospital, Beijing; Croatia: Center in
REFERENCES


MASA — actions louder than words

The SAMJ has frequently been the recipient of correspondence and telephone calls from irate members wanting to know what the Association is doing about a variety of issues that are of fundamental concern to the profession — concerns that have often generated vigorous correspondence in our 'Letters' section. The SAMJ has therefore deemed it appropriate to look into the position of the Association in respect of these issues, and to provide its readers with up-to-date information on them.

One such concern has centred around the absence of an appeals procedure against SAMDC disciplinary committee hearings. The MASA has pursued this matter with the SAMDC over the years, and was recently able finally to reach agreement with the Council on the need for the right of appeal to the Supreme Court on the substance (rather than just the procedure) of the hearing and the verdict. This agreement, which represents a major breakthrough for the profession, must now be taken further through the channels necessary to convert it into legislation.

Secondly, the MASA has successfully negotiated an above average increase in the rates of RAMS Scale of Benefits for 1994, and has further won the retention of direct payment of medical aid claims to the doctor, all of which represent the benefits of the recently established formal negotiation agreement between the MASA and RAMS. RAMS has further agreed to grant an additional rate increase in about 6 months, provided that the doctors achieve savings for the societies through the containment of prescription and hospitalisation costs. Although it bears repeating that the doctor is not the sole or even the main cause of the high cost of medical care, there is no doubt that both these areas present good opportunities for cost-containment without threatening the quality of care offered to the patient, and the offer of incentives by RAMS represents a sensible way of addressing the escalation of health care costs in South Africa.

Thirdly, the government has come up with additional funds earmarked for certain 'disadvantaged' categories in the public service. The MASA has lobbied strenuously for a sizeable portion of those funds to be allocated to the hard-pressed doctors in state employment, for it is well recognised that state-employed doctors have consistently got the short end of the stick over the years when it came to remuneration, something to which the Association has always been sensitive. The MASA's hand was strengthened recently when it was recognised as the negotiating agent for the medical profession with the State and, in respect of the additional funds already mentioned, the negotiating team is confident that a portion of these funds will go towards addressing certain structural inequities of the past.

Fourthly, significant progress has been made with regard to the vexed question of pharmacists being allowed to diagnose and treat medical conditions. Following intensive legal research, legal consultation and political lobbying, the MASA obtained the agreement of the Parliamentary Committee on Health that the proposed amendments to the Pharmacy Act which would have extended the authority of the pharmacists to practise medicine would have been legally intolerable and morally unjustifiable. The MASA has argued inter alia that any amendments authorising pharmacists to diagnose and treat disease without SAMDC training and registration would be ultra vires, and would any related regulations by the Medicines Control Council. The Association is sufficiently confident of its position in this regard to be ready to take the matter to the Supreme Court, if the need should arise.

Finally, the MASA is marching in step with the sociopolitical transformation that is unfolding in our country. It has forewarned political affiliation, and has positioned itself as a national professional organisation dedicated to serving the medical fraternity and the entire community. It was in recognition of this ongoing transformation that the MASA was recently invited to join the Confederation of African Medical Associations and Societies (CAMAS), an organisation originally formed as a protest against apartheid and the MASA. The Association views its newly found relationship with CAMAS as an opportunity for future co-operation with other African countries in such areas as medical research, collection and dissemination of information, and the promotion of appropriate health care through our respective governments.

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The South African Bone Marrow Donor Registry

Allogeneic bone marrow transplantation is the preferred form of therapy for patients with aplastic anaemia or severe immunodeficiency disease and is increasingly being used in leukaemia, the lymphomas and myeloma. In these and other malignancies, escalation of chemotherapy to supralethal levels can be achieved with relative safety by means of autologous procedures, preferably in conjunction with recombiant human growth factors. However, currently available conditioning regimens are not universally effective in the eradication of minimal residual leukaemia, so that interest centres on the generation of graft-versus-leukaemia (GVL), an immunologically mediated phenomenon associated with allografting. Unfortunately, this effect is associated only with the more severe instances of graft-versus-host disease (GVHD), resulting in unacceptable rates of morbidity and mortality.

The barrier to allogeneic transplantation lies in a series of cell surface glycoproteins, collectively referred to as the major histocompatibility antigens. In humans, these are designated the human leucocyte antigen (HLA) system, which is encoded by a number of genes located on the short arm of chromosome 6 and comprises two major groups. The first, designated class I, HLA-A, B and C loci, is extremely polymorphic. In 1991, there were 22, 50 and 11 alleles reported respectively, whereas are identified by standard serological methods. The second, or class II antigens, are less variable, with 18, 9 and 6 alleles described for the DR, DQ and DP loci; many of these can only be detected by DNA typing. Gene amplification by means of the polynucleotide chain reaction (PCR) and subsequent charac-
editorial of the alleles by hybridisation with complementary DNA sequences or probes, are the preferred methods, because serological techniques are not reliable in an estimated 25% of cases.8,9

In general, the results of transplantation are better with fully histocompatible donors and recipients, although in the case of solid organs some discrepancy can be tolerated and graft failure reduced by a variety of immunosuppressive drugs. In contrast, bone marrow transplantation is critically dependent on HLA identity, because even single amino acid differences between antigens are associated with rejection or the development of GVHD. One way to overcome these complications is by autografting. Alternatively, an HLA-identical sibling is used as the donor. The clustering of loci in the system on the chromosome generally results in their inheritance as a complete HLA-haplotype.

At present, 60 - 70% of patients needing a transplant have a suitable sibling, but DNA-typing has made it possible to identify unrelated HLA-identical individuals who can serve as bone marrow donors.10,11 In these situations, the incidence of graft failure as well as both acute and chronic GVHD are increased. Protracted and intense immunosuppression,12 including the removal of cytotoxic T-cells from the bone marrow,13 reduces the incidence and severity of the latter complications. Nevertheless, this new therapeutic option is gathering impetus and, over the last 5 years in Europe and North America, has led to the establishment of registries for this purpose. Initially, volunteers were recruited from apheresis programmes because they had already been tissue-typed, but increasing attention is being directed toward blood donors and the general public.14 Interested individuals sign a statement of intent to donate bone marrow and a blood sample is collected for typing of the HLA-A, B, C and DR alleles by serological methods. The results are entered into a computer database. When needed, a search is undertaken to identify potential HLA-identical individuals, after which DNA-typing of the DR, DQ and DP loci as well as mixed lymphocyte culture testing are used to confirm histocompatibility. A pool of between 100 and 10 000 donors of the same race as the recipient is needed in order for there to be a realistic chance of finding a matched donor. In any search, the likelihood varies, depending on whether the HLA-haplotype of the patient is common or rare.15

To increase the number of potential donors, national registries co-operate through the World Marrow Donor Association, thereby providing access to some 800 000 potential donors.

Should there be more than one registry in a country, co-ordination is ensured by the hub centre. A prerequisite for such a designation is an intimate association with an internationally accredited transplantation group on the one hand and the availability of the latest tissue typing technology on the other. In South Africa, these criteria are met by the Provincial Laboratory for Tissue Immunology, which has a bone marrow donor registry, together with the University of Cape Town Leukaemia Centre and the Department of Haematology at Groote Schuur Hospital. The justification for such an undertaking is the fact that allele and haplotype frequencies of the HLA system differ considerably between races throughout the world16 and at present most donors are white. For instance, the frequency of the most common haplotype in blacks, A30, B42, and DR3, is 7,5%; in whites the frequency of this haplotype is only 0,02%. Therefore, a particular problem exists for sub-Saharan Africa, because matched donors for non-white patients are difficult to find.

Despite the fact that there is undoubtedly an increased risk of severe acute and disfiguring chronic GVHD after unrelated matched transplants,17 some recent results obtained after T-cell depletion of bone marrow prior to transplantation have been encouraging.18 In patients with leukaemia, there is a higher risk of graft failure with well-matched unrelated donors, compared with HLA-identical siblings.19 The incidence of GVHD and long-term survival rates of the two groups were comparable. However, such figures do not reflect the poor quality of life which results from debilitating GVHD. Because the incidence and severity of this complication increase with the degree of mismatching,20 it follows that meticulous donor selection is mandatory. Alternative HLA-identical donors from within the family and the use of matched unrelated volunteers are relatively recent therapeutic approaches, and their use is tempered by an appreciation of the devastating side-effects that may occur.

Certain studies21,22 examining donor recruitment include the question of their commitment to donate bone marrow when requested and the estimated 10% annual loss of this population through relocation. In North America, specific problems have been encountered in motivating organ donation among minority groups; objections were mostly of a cultural or religious nature. Because the local registry targets non-whites, a particular effort has been made to anticipate the question of informed consent by having recruiters who are fluent in Xhosa, as well as Afrikaans and English. Comprehensive information leaflets, accompanied by a statement of intent to donate, are available. Lectures have been given to groups in the private sector, colleges and a variety of other institutions to explain the logistics of the donation itself and the risks to the donor, objectively balanced against benefits to the patient. To date, approximately 10% of those addressed have responded favourably. After the blood has been tissue typed, this information is made available to the donor on a card that also carries the address of the registry. Like most registries, the initial search will be without cost, but further investigations to confirm matching will be billed to the recipient.

More information is required to answer questions regarding the definition of an acceptable unrelated bone marrow donor, refinement of T-cell depletion programmes and post-transplant immunosuppressive regimens. Despite these reservations, preliminary results are such that the effort of establishing and maintaining a registry is entirely justifiable.12,16

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ISSUES IN MEDICINE

Keeping the prescribing pharmacist at bay

The campaign to legalise the rendering of medical care by pharmacists has been thwarted by the Parliamentary Joint Committee on Health. The Pharmaceutical Society of South Africa in its submission concerning certain amendments to the Pharmacy Act, which would have created the scope for such practices, noted that the MASA presented expert legal and medical opinions in support of its arguments that the proposed amendments would have been legally intolerable and a public disservice. The basis of the MASA's strategy was to gain a lobbying voice and insist on maintaining the professional image of the medical profession by refusing to fight the matter through the media. The MASA is confident that the Medicines Control Council (MCC) will now also not introduce draft regulations to the same effect in terms of the Medicines and Related Substances Control Act. Legal counsel advises that this would be ultra vires, and that MASA should appeal to the Supreme Court if the MCC introduces the regulations.

During 1993 the pharmaceutical profession intensified its campaign for authorities to treat and diagnose medical conditions. Advertisements appeared in all possible media. The campaign became more of a reality with more and more pharmacists applying for special permits to this effect from the Department of National Health and Population Development (DNHDP). The MASA intervened immediately and the permit 'issue was partially resolved when the Director-General of the DNHDP placed a moratorium on the issuing of permits, agreeing to issue them personally and only on merit. However, in September the Pharmacy Amendment Bill was published. Only 4 days were given to interested parties to prepare comment on the Bill. After having presented verbal and written representation to the Parliamentary Joint Committee on Health on two occasions, the MASA succeeded in convincing them to withdraw certain amendments which failed to delineate the role of pharmacists.

Some of the main points of criticism of the Bill related to the fact that the legislation would have entitled pharmacists to diagnose and treat patients. Furthermore, vague and unspecified provisions were proposed to broaden the role of the pharmacist. This was totally unacceptable, as it would have created legal uncertainty and a criminal offence.

The MASA also argued that allowing pharmacists to render clinical health care would not alleviate the burden on the State and make health care more accessible — more than 80% of pharmacists are in the private sector and in 24% of the magisterial districts there is no pharmacy.

The MASA is of the opinion that the Pharmacy Amendment Bill was in fact published in an attempt to legalise' draft regulations in terms of the Medicines and Related Substances Control Act, which was published 3 months earlier. These draft regulations, which are currently before the MCC, have the implicit intention of allowing pharmacists to render clinical medical care. The MASA intends to appeal to the Supreme Court if the MCC proceeds with their implementation. It is envisaged, inter alia, that pharmacists would be allowed to diagnose and treat patients, and to prescribe medicines up to Schedule 5 — in some instances even up to Schedule 6. This is not allowed in terms of the Pharmacy Act and can therefore not be effected through administrative regulations not debated in Parliament.

The draft regulations further envisage that the SA Pharmacy Council would accredit training courses for pharmacists. This, too, is prohibited in terms of the Medical, Dental and Supplementary Health Service Professions Act, which provides that only the South African Medical and Dental Council (SAMDIC) may approve the training of persons to 'diagnose, treat or prevent any physical or mental defect'. Accreditation of these courses by the SA Pharmacy Council would therefore contravene the above Act.

Statements by spokespersons of the pharmaceutical profession that pharmacists do not need more than 1 week of training are blatantly untruthful and arrogant. The medical profession promotes continued education even after 7 - 12 years' intensive practical and academic training.

Fragmented control over pharmacists and doctors authorised through separate statutory councils to perform the same actions and carry the same responsibilities, will lead to professional inconsistencies. The South African law does not tolerate double standards. The principle of the 'reasonable man' prevails and doctors are judged against available fields of expertise. A pharmacist giving clinical medical care will be judged against the skills of a reasonable general practitioner and not those of a reasonable pharmacist. The MASA has numerous examples of misdiagnoses and maltreatment by pharmacists that caused permanent damage and in some cases were fatal.

MARJOLEEN VAN WYK
The South African Bone Marrow Registry (SABMR) and allogeneic bone marrow transplantation

Paul Ruff, Terry Schlaphoff, Ermette du Toit, Anthon Heyns, on behalf of the Board of the South African Bone Marrow Registry

In 1939 Osgood et al. reported infusing bone marrow into patients with severe aplastic anaemia without any clinical benefit. Roess and Coulter attempted to reconstitute marrow function in irradiated dogs by marrow infusions. Both failed because of insufficient irradiation to produce immunosuppression necessary for engraftment. In 1957 Donnall Thomas and co-workers showed that marrow can be collected, stored in significant quantities and safely administered. Marrow transplants remained unsuccessful, however, although patients with refractory leukaemia given sublethal irradiation recovered after marrow infusion from identical twins. Allogenic marrow transplants usually resulted in failed engraftment, or engraftment followed by lethal graft-versus-host disease (GVHD). Further discoveries were the HLA complex and transplantation antigens, and that successful allogeneic engraftment depends upon donor/recipient histocompatibility.

These studies pointed the way for human marrow grafting using HLA-matched sibling donors. Thomas et al. showed that it was possible to cure leukaemia, aplastic anaemia, thalassaemia and inherited marrow disorders using a histocompatible sibling donor. The finding that methotrexate and subsequent immunosuppressive agents such as azathioprine, cyclosporine and tacrolimus, reduced GVHD as well as graft failure, improved results of marrow transplantation.

Haematopoietic growth factors

The discovery of haematopoietic growth factors, granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage-colony-stimulating factor (GM-CSF) in the 1980s had a major impact on marrow transplantation. CSFs stimulate haematopoietic progenitor cells, permitting rapid proliferation and repopulation of marrow by engrafted cells. Recombinant DNA technology enabled CSFs to be synthesised for clinical use.

The physiological effect of G-CSF in mobilisation of haematopoietic stem cells into peripheral blood enabled peripheral blood stem cell harvesting to replace marrow harvesting to obtain stem cells. A cell separator harvests stem cells from peripheral blood, obviating general anaesthesia to aspirate marrow. Donors are given a 5-day course of 5 - 10 μg/kg subcutaneous G-CSF before the stem cells are harvested. It takes 4 - 6 hours to obtain sufficient cells for the infusion.

Graft-versus-host disease

Although some early transplants were successful, problems occurred due to graft failure, rejection and acute GVHD. Some patients receiving marrow from siblings developed a potentially fatal syndrome of an erythematous desquamating rash, watery diarrhoea and jaundice.

The most successful transplants, with minimal rejection and GVHD, are between identical twins, i.e. syngeneic transplantation.

Immunosuppressive agents

Immunosuppression, first developed in renal transplantation, was then applied to marrow transplantation. Immunosuppressive agents included azathioprine, cyclosporine, antithymocyte globulin, antilymphocyte globulin and later tacrolimus, antiCD25 (T-cell receptor) monoclonal antibodies basiliximab and daclizumab, and mTOR (mammalian target of rapamycin) inhibitors, sirolimus and everolimus.

Murine anti-CD52 monoclonal antibody, Campath-1G, when added to the harvested stem cell bag, removes almost all lymphoid cells, limiting GVHD although increasing the risk of leukaemia relapse. Humanised anti-CD52 monoclonal antibody, alemtuzumab, used intravenously before infusing...
stem cells, reduces murine protein anaphylactic reactions and the development of human anti-mouse antibodies (HAMA).^4

**Graft-versus-leukaemia effect**

A major reason for success of allogeneic transplantation in myeloid and possible lymphoid malignancies was the suppressive effect of donor T lymphocytes and NK cells on the underlying malignancy. This graft-versus-leukaemia (GVL) effect has to be balanced against the risk of GVHD. Mild GVHD with GVIL is ideal whereas severe GVHD is undesirable. Patients receiving T-cell-depleted stem cells or syngeneic transplantation have a higher risk of relapse of their underlying disease.^

**Lack of sibling donors**

As donors for an allogeneic stem cell transplant require an exact HLA Class I (A, B, C) and Class II (DR, DQ) match with the recipient, most patients requiring transplantation do not have a readily available donor. Siblings are the usual source of donors, being the only available matched family members. Today, with smaller families, only about 10% of patients have an HLA-matched donor. There is a 3% chance of finding a suitable donor in the extended family, e.g. cousins. Transplantation with 1-locus or even 2-loci mismatches from a family donor can be performed, but the risks of graft failure and GVHD increase. Haplo-identical transplants from parents to children play a role when no donor is available but are risky.

Owing to this lack of donors, and the expansion of allogeneic stem cell transplantation in malignant and non-malignant diseases, an alternative source of donors had to be found.

**Matched-unrelated-donor stem cell donors**

Two alternative non-family sources of stem cells are now used, namely matched unrelated donor (MUD) transplants and umbilical cord blood transplants.^

MUD registries have grown in most developed countries. Racially homogeneous societies such as Scandinavia lend themselves best to such registries, as HLA genes are largely ethnically based. Most European registries have donors of Caucasian origin, making access to donors of Asian and African origin difficult to obtain. The ethnically diverse population of the USA is the best source of donors throughout the world, but high costs and strict National Marrow Donor Program (NMDP) regulations limit international access to US donors. In August 2006 South Africa signed a co-operative agreement between the NMDP and the South African Bone Marrow Registry (SABMR).

Donor registries co-operate and their linked databases enable patients to have access to donors worldwide. At the time of writing in December 2007 there were 59 stem cell registries and 40 cord blood registries worldwide with access to 12,02 million registered donors.

**Umbilical cord blood transplantation**

Umbilical cord blood, the other alternative source of stem cells, is useful for children and for small adults (under 50 kg) and furthermore requires a less stringent HLA match. Stored umbilical cord blood does not rely on donor presence and current good health. Once cord blood has been shown to be HIV, hepatitis B and C virus-free, the donor is guaranteed. Living MUDs may change their minds, move towns, die or seroconvert. The HIV epidemic, limited resources and lack of government support have resulted in few moves to develop umbilical cord banking in South Afrika.

**Bone marrow transplantation in South Africa**

Bone marrow transplantation in South Africa was developed at the University of Cape Town from the mid-1970s, and the University of the Witwatersrand in the 1980s.

**South African Bone Marrow Registry (SABMR)**

The SABMR was started at University of Cape Town by Professor Ernest du Toit with help from Professor Peter Jacobs in early 1990. It is a non-profit organisation (Reg. No. 004-300 NPO) that co-ordinates the provision of matched unrelated stem cell donors for South Africa, and is the contact for countries seeking unrelated donors in South Africa.

The SABMR was established in the Laboratory for Tissue Immunology (LTI) in Cape Town. This was an extension of the LTI functions, which was initially HLA typing for organ transplantation since Chris Barnard’s first heart transplant in 1960s. The European Federation for Immunogenetics accredited the LTI as an HLA-typing centre for unrelated marrow transplantation in 1999.

The first donors were recruited in 1991. By May 1992 there were 210 donors on the registry, and by 1993 there were 554, of which 482 were HLA-ABDR typed. Owing to lack of funding and donor awareness, the SABMR grew more slowly for the next 7 years. Donor awareness was increased with the help of the National Blood Transfusion Service (NBTS) and two donor funding organisations, the Darren Serebro Foundation in Gauteng and the Sunflower Fund in Western Cape. The Darren Serebro Foundation, which started in August 1996, recruited over 20,000 donors, mainly in Gauteng. The Sunflower Fund formed in 1999 was inspired by the struggles of Chris Corlett and Darren Serebro against leukaemia. The aim of the two organisations is to raise financial support to increase the number of stem cell donors in South Africa. The Sunflower Fund now has the national responsibility for raising funds for tissue typing of unrelated donors, which had resulted in an increase in the SABMR donor database size to 63,123 by December 2007 (Fig. 1).

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In the worldwide database of over 12 million, 70% are HLA-ABMR typed. Unfortunately only 6% of the potential SABMR donors have been HLA-DR typed (Fig. 1). This deficiency is now being addressed by the SABMR.

Lack of black donors makes MUD transplantation problematic in South Africa, with very few recipients of African origin. This problem is exacerbated by the lack of donors of African origin on European registries available to South African patients. The NMDP is a potential source of donors of African origin, although African-Americans are mainly of West African origin with different HLA typing frequencies to Southern African blacks.

Following the recruitment of the first donors, the World Marrow Donor Association (WMDA) recognised the SABMR as the HUB centre for South Africa in September 1991. The WMDA is a voluntary organisation of representatives of blood stem cell donor registries, cord blood banks, other organisations and individuals with an interest in haematopoietic stem cell transplantation. It provides a forum for discussion of issues regarding the clinical use of haematopoietic stem cells from unrelated donors across international boundaries and for formulation of guidelines on logistics, quality control, ethics, finances, information technology and registry accreditation.

Bone Marrow Donors Worldwide (BMWDW) situated in Leiden, The Netherlands, is a voluntary collaborative effort of stem cell donor registries. BMWDW collates donor information worldwide. This database contains details of over 12 million donors, including more than 63 000 from South Africa, and is available online to all participating registries. This allows all registries to search for donors when there is no local donor available.

**Matched unrelated donor transplants in South Africa**

The first MUD transplant in South Africa was performed at the new University of the Witwatersrand Donald Gordon Medical Centre in Johannesburg in June 1997. Bone marrow was harvested in Belgium and flown to Johannesburg. In 1999 the first SABMR donor provided peripheral blood stem cells to a South African patient. In 2003 the first black South African donor provided stem cells for an unrelated patient and the first unrelated cord blood transplant was performed. At the time of writing, a total of 125 patients had received first MUD transplants in Gauteng and the Western Cape (Fig. 2). Of the donors 32 were from the SABMR and 93 from international registries, mainly in Europe (of the latter 6 were cord bloods). In addition 7 SABMR donors supplied stem cells to patients in Germany, Italy, Portugal, The Netherlands, the UK and the USA.

**Conclusions**

Haploidentical bone marrow and peripheral blood stem cell transplantation has become an important treatment of haematological malignancies, first acute and chronic myeloid leukemia and myelodyplasia, lymphomas and even multiple myeloma. Its role in chronic myeloid leukemia has diminished considerably since the advent of imatinib.

It also plays an important role in non-malignant haematological diseases, first aplastic anemia and now haemoglobinopathies, thalassemias and storage diseases.

Modern tissue typing and immunosuppression, and better supportive care including newer antibiotics, antifungal and antiviral agents, have reduced mortality and morbidity.

Alternative sources of stem cells, including MUD and umbilical cord blood transplantation, have widened the availability of donors throughout the world. The growth and development of the SABMR is essential in our quest for donors, especially for patients of black African origin, and needs support from the public, donor funds, universities, private corporations and the government.

Immunohaematopoietic stem cell transplantation in South Africa
- The first 40 years – An experimental and clinical model for approaching restorative medicine

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Abstract
Hippocratic teaching exalts literally centuries of ethical principles in medicine. The foremost of these being to heal and do no harm – an ideal for which all clinicians strive but advances are seldom – if ever – entirely risk free. The way in which such balance is achieved presupposes our remaining perpetual students seeking to couple current knowledge with a clear perspective of perceived benefit versus defined hazard. Wisdom to this degree is found in few individuals but, nevertheless, as an ideal, serves to define the true scientist who, tenaciously, uncompromisingly and over prolonged periods systematically completes basic research for subsequent translation into clinically relevant practice.

Nowhere are these concepts better illustrated than in the burgeoning field of stem cell biology. Media sensationalism, commercial or monetary opportunism and self-aggrandisement continue to confuse the vital task of advancing this aspect of medicine but doing so within moral and religious constraints. A further impediment to progress is failure to distinguish between embryonic and adult options. In the former the steps closely replicate physiology and the totipotentiality of the inner cell mass is the basis for the parallels in reproductive and therapeutic cloning. Intrinscic to these techniques must be definitions as to when life begins and debates to bring order to widely divergent viewpoints extend all the way to the American Congress! The possibilities are enormous but require safeguards to be generated from dispassionate debate primarily between scientists and ethicists rather than politicians. These issues are not our immediate concern.

Rather, in contrast, starting material from already formed organs has restricted capacity for differentiation and proliferation with a different challenge to resolve – can one source give rise to another function? To grasp this

nettle consider immunohaematopoiesis where comprehensive repair of the irreversible aplasia can be effected using only a minute representative inoculum from a matched donor. But can altering the microenvironment change the incoming phenotype to undergo modification needed to restore damage when transferred to a quite different tissue? This phenomenon, described as plasticity, is tantalising but not yet a clinical reality. Some of these controversies were explored at a Cape Town workshop held in November 2005 giving rise to a study group which will hopefully sustain the initiatives with appropriately regulated protocols to encourage cross-disciplinary development in South Africa.

Bone marrow is obviously the logical starting point on which to build. Here a wealth of meticulously chronicled and relevant information from a wide range of experimental animals, supplemented by clinical usage, is available. For example much is known and precisely defined in autografting, events in histo-compatible siblings and, more recently, what happens when matched unrelated volunteer donors are used. Thus a solid foundation characterises fundamental aspects of these procedures incorporating the immunogenetics necessary to continue unravelling early cellular and molecular events that occur in the recipient. Stated differently these phenomena provide the essential reference from which carefully stated questions can direct, innovatively, the activity of working parties to test what happens to the explant growing in a foreign anatomical site – the fundamental theme of this emerging field. In context it can be noted that many of the central aspects are already locally established and continue to benefit from international collaboration. The phases leading to the current status of this single centre team are summarised to illuminate how haematology can play a role in testing both principles and practical aspects of widening indications for these interventions.
Introduction

The emerging role of stem cells in biology continues to accelerate thereby focusing attention on terminology. Two broad categories of donor material exist. Their fundamental behaviour is widely divergent and continues to generate debate between scientists, philosophers, ethicists and, increasingly but a lot less helpful, politicians!

HUMAN EMBRYONIC ORIGIN is considered here only to clarify fundamental features. The defining property is a capacity to produce all the tissues that make up a complete individual where the first step is fusion of ovum and sperm to generate the zygote. During passage down the fallopian tube and early phases of implantation in the uterine wall the morula gives rise to blastocyst in which recognisable inner cell mass has pluripotentiality but lacks the capacity to form extra-embryonic structures. During gestation three germ layers form known as the ectoderm, endoderm and mesoderm and these constitute the blueprint for a complete offspring.

It follows that capacity to recapitulate physiology in vitro or in cultures is momentous. In scientific terms recovery of the blastocyst and implantation into a surrogate uterus is feasible as seen in meticulously selected cases generally at fertility clinics. A variation is reproductive cloning where, through the process of somatic cell nuclear transfer, the original or haploid nucleus from the oocyte is removed and replaced with a mature counterpart where the chromosomes originate from the patient. The therapeutic equivalent is an interesting variation where the early blastocystic stage is cultured in vitro with differentiation being driven towards multipotentiality and exemplified by muscle, neurons or blood depending on modulating influences in the medium. (Figure 1)

These somewhat different options have profound moral and religious overtones encompassing criteria for when life commences and predicated the need for inclusive and sensitive monitoring at every stage to avoid even the slightest abuse.

**Figure 1: Contrasting stem cell methodologies**

<table>
<thead>
<tr>
<th>Physiology</th>
<th>Oocyte + sperm</th>
<th>zygote</th>
<th>blastocyst</th>
<th>uterine implantation</th>
<th>Normal offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reproductive cloning</td>
<td>Enucleated oocyte + adult cell nucleus</td>
<td>nuclear transfer</td>
<td>nuclear transfer</td>
<td>uterine transfer</td>
<td>Cloned Offspring</td>
</tr>
<tr>
<td>Therapeutic cloning</td>
<td>Enucleated oocyte + adult cell nucleus</td>
<td>nuclear transfer</td>
<td>harvested embryonic stem cell</td>
<td>Cultured in vitro to yield mature cells</td>
<td></td>
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<tr>
<td>Adult cell cloning</td>
<td>Haematopoietic stem cell</td>
<td>expand ex vivo</td>
<td>seed to new environment</td>
<td>Plastic response to altered phenotype</td>
<td></td>
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</table>

Indeed the recent South Korean claims highlight the sensitivity of this area. Enormous restraint, transparency and responsible reporting are mandatory for constructive and sensible regulation but this needs to be by knowledgeable peers working in the field if future benefit is not to be denied.

ADULT SELF RENEWING populations provide the counterpart and are found in all organs where they provide for natural repair as in wound healing or sustaining blood formation from the bone marrow. The latter has been studied extensively and is the basis for autologous or allogeneic immunohaematopoietic grafting. Long years of intensive study have established that numbers with repopulating potential are relatively small but, can be mobilised into the circulation for recovery: however keep in mind their protracted exposure to environmental toxins and the accumulation of genetic injury over the lifetime of the donor. Despite such drawbacks preliminary data support the possibility that they can be expanded ex vivo and, furthermore, manipulation of culture conditions subtly alter the phenotypes. If correct, the tantalising concept arises in which such multipotential progenitors actually have less circumscription that long espoused as conventional dogma but rather that anatomical boundaries can be transgressed. For example incorporation of nominally blood forming precursors into heart muscle after infarction or, cartilage, ligaments and even nerves, aiding recovery, may be possible. Whether such plasticity exists at all or, in the diametrically opposed viewpoint, is a reality proposes the existence of a living reagent with wide ranging restorative properties. Not surprisingly there is support for intensive study to meticulously separate fact from fantasy. This approach is somewhat less emotion-laden than the use of pre-implantation material but, is nevertheless, every bit as relevant and, accordingly, a foundation must be established in soundly based, rigorous tested and confirmed science. Unless restraint is exercised there is a risk of generating premature and unjustified expectations which will tarnish all efforts by reputable investigators to proceed constructively. This charge has been given national priority by convening a study group under the aegis of Stellenbosch University jointly with the South African Medical Research Council. Here one group will seek to consolidate experience that has accumulated with international collaboration over the last 35 years and continue to explore the enigmatic haemangioblast, or even earlier forms, in restorative medicine. The events leading to the current status are therefore summarised as a record as to how this point was reached.

Bone marrow transplantation as a model

EXPERIMENTAL HAEMATOLOGY, using a murine system, demonstrated reappearance of blood formation in a medullary cavity rendered aplastic by lethal irradiation and showed that this was not due to plasma factors but cell mediated.1 Elegant studies continue to define fundamental aspects of homing to special sites known as niches, where particular environmental influences dictate differentiation in what is then described as the haematopoietic inductive microenvironment.2 Localisation of precursors in relationship to marrow trabeculae is constant and dictated by a complex system of interactions controlled by stromal ligands in the form of adhesion molecules or cytokines recognising cognate receptors on primitive progenitors.3 Complimentary observations that a unique relationship exists between supporting mesenchyme and early committed lineage find support in the culture system described by Professor Michael Dexter. Particularly relevant is an in vivo splenic assay where the content of the growing colonies vary according to the particular site in this organ where they eventually develop.4 In broadest context these interactions have stimulated developments widely tested including the use of higher primates. It is not unreasonable to anticipate that adult progenitors have an intrinsic capacity to undergo phenotypic remodelling depending on how they are stimulated and, in this way, participate in repair of muscle, ligaments and perhaps even neurons.5 It might be asked, somewhat rhetorically, if quiescent populations exist in solid organs, presumably dedicated to just this task, why are extrinsic sources needed? This, and other inconsistencies, await systematic study.

CLINICAL STUDIES were pioneered by Professor E Donnall Thomas6 and generations of his research fellows drawn from every corner of the globe. Although a number of additional groups were at about the same time involved in developing these procedures it would be invidious not to recognise the contributions of Professor George Santos at Johns Hopkins, and the outstanding radiobiologists such as Dirk van Bekkum, in the Netherlands.
Development of the Cape Town programme

The seed is sown

A number of sequential steps characterised first the introduction and then the systemic consolidation of the infrastructure which preceded the first bone marrow allograft in South Africa at Groote Schuur Hospital in 1972.

1964 saw the first attempted procedure in this country. A 23 year old male medical student with severe aplasia received a somewhat arbitrary volume of anticoagulated aspirate from a brother. Engraftment did not occur and he died as a result of neutropenic septicemia. In retrospect this is not surprising since little was known about histocompatibility and even less about graft composition or immunosuppression. This event, combined with an ever-expanding literature on related topics including human leukocyte antigens system, reports of success in leukaemia and Fanconi or aplastic anaemia made an indelible impression during fellowship years spent with Professor Clement A Finch in Seattle where there was close contact with the multidisciplinary program directed by Dr Thomas.

Inbred rabbit strains were used rather than mice

On return to this country it was a considerable honour to be appointed as Foundation Professor of Haematology at the University of Cape Town. Fortuitously, just at this time, Professor Christiaan Neethling Barnard had carried out the first successful human heart transplant and ignited interest from Professor Eugene B Dowdle and Dr M C Botha in providing immunologic assays for the histocompatibility testing. Not surprisingly many were caught up in this new field and the fledgling department started funnelling all resources and energy into marrow grafting as the major direction for the future. From the first day encouragement and support was abundantly available from faculty, staff and colleagues making it possible, somewhat painstakingly at times, to create the infrastructure culminating in the first successful such procedure within two years. This was not a random event but rather the starting point of a carefully planned and projected long-term core activity.

Methodology in evolution

Laboratory processing was set up by collaboration with Professor Bruno Speck and Dr Alois Gratwohl in Basel in which, rather than using mice, inbred rabbit strains were selected to refine the details of each step. Harvesting femoral marrow, as a surgically sterile procedure under general anaesthetic, and the preparation of a monocellular suspension was perfected using a series of stainless steel screens of decreasing pore size. After radiotherapy of recipients, skillfully provided by Dr Basil Shepstone and Professor Rossall Sealy, autografting was used to compare intravenous infusion to block re-implantation but showed no difference. This required establishment of a vivarium and the considerable skills of Mr Graham Manual as a cardinal member of the team are remembered with appreciation. Graft monitoring was primitive using only mononuclear numbers and all efforts to antigenically match pairs was unsuccessful. The alternative measurement, shifted to clonogenic assays and started in the Heath-Robinson constructed carbon dioxide incubator built by Dr Joan Parker. Rapidly erythroid, granulocyte and eventually mixed colony growth was standardised as the reference point for quality control and it was possible to demonstrate a linear relationship between these measurements and engraftment. The scene was set to translate laboratory results to patients.

The clinical programme

Heart breaking frustration occurred in a number of areas during this first decade. The need to interact with more experienced colleagues in other parts of the world was impeded by working in a country blanketed by an intense academic boycott directed at the apartheid policy. Despite this a steady stream of investigators from most of the major centres found ways of visiting and, reciprocally hosted ourselves in initiating and consolidating this activity. Benefit undoubtedly accrued from the local cardiac and renal success.
Immuno-haematopoietic stem cell transplantation in South Africa

Figure 2: The cell separator
The later generation cube spectra with world authority Professor Jean Porter Hester updating methodology with SPH Lucille Wood and SPH Louise Abrahams.

Apheresis technology provided a means to discontinue the supply of blood and single unit platelets in glass bottles! Replacement of this rather quaint but obsolete service was made possible by recognising the superiority of the cell separator and the IBM 2990 was secured by donation. Over the ensuing years this methodology became standard and gradually permeated the commercial transfusion services first in the Western Cape and subsequently in other provinces. This period was stimulated by a visit from Professor Jean Porter Hester and this association continues actively today. (Figure 2) A major benefit was the ability to recover the corresponding population having engraftment potential from the peripheral blood which became the norm and further refinement is a current priority.

A further innovation was the creation of a dedicated platelet donor panel made up of doctors and staff in the department and unselfishly from volunteers throughout the Groote Schuur Hospital at every level from gardeners through janitorial staff to senior consultants. This philosophy underscored the approval of the new activity and serves as a reminder of the spirit de corps found in the corridors of the old hospital.

Unfractionated marrow was used initially requiring conventional immunosuppression with corticosteroids and methotrexate. (Figure 3) Rejection and acute as well as chronic graft-versus-host disease paralleled world experience and, while as elsewhere some success was achieved, the morbidity and the mortality of these complications exacted a heavy toll on nursing and medical staff alike. At this point the team became aware of the need to add a staff psychiatrist, a dedicated social worker and physiotherapist, all innovations remaining central to the current programme more than 30 years later.

Alternative sources include umbilical cord blood which is available primarily on a dedicated basis within families. Access to this product is further coordinated through the South African Bone Marrow Donor Registry and our membership to Eurocord. This is a valuable resource and may well be a further productive area for study in non-haematopoietic mediated tissue repair.

Whether, in the South African context, there is a place for banking remains far from reality although a local working party was established some years ago to explore this possibility: (Jacobs and Wood unpublished).

Much of the early hazard attributable to neutropenia, compounded by unavoidable immunosuppression, created substantial morbidity and mortality. Here one continues to reflect on the extraordinary degree of encouragement from the surgeons including access to their specialised beds and nursing expertise in the eleventh block. The challenge of overcoming potentially or sometimes lethal complications reinforced the need to draw on expertise scattered throughout the School. This led to creation of the multidisciplinary Haem Team which, to this day, routinely includes pulmona, cardiac, renal and infectious disease consultants in the moment-to-moment management of those complex clinical problems.

A series of rather distinct, albeit overlapping periods, can be recognised in this historical perspective:

Figure 3: The first bone marrow transplant in South Africa
The two teams simultaneously harvest from sternal and iliac crest. The donor is anaesthetised. Each syringe of heparinised marrow-rich blood is processed by a trained technologist.
Unrelated donors may be needed where siblings are not available. To overcome this obstacle we formed the South African Bone Marrow Donor Registry jointly with Dr Arthur Bird as Medical Director of the Western Province Blood Transfusion Service and Professor Ernest du Toit as Head of the Provincial Laboratory for Tissue Immunology. Scope continues to expand and, as the Hub centre there are links with the corresponding European Organisations and the American National Donor Program where the Constantia team is designated as a transplant and harvest centre. There has gradually been increasing recognition of these activities that found expression in the 6th International Donor Registry Conference from 26th May – 27th May 2006 in Cape Town.

Cyclosporin A became available as a result of cooperation studies with Professor Jean Borel in Sandor in Switzerland. Years of a fruitful collaboration explored the role of this unique immunodepressant in the postgraft period but the anticipation of complications disappearing was unfortunately short lived. Undoubtedly incidence and severity of these immunologically related phenomena decreased but they remained a major challenge to the investigator. Monoclonal antibodies became available as a result of a still-active association with Professor Herman Waldmann and Dr Geoff Hale first in Cambridge subsequently in Oxford. Here, as part of the Campath Users Group, there was an opportunity to investigate the in vivo use of these immunoglobulins for T lymphocyte depletion. A previously untested modification, namely their admixture to the harvest in-the-bag without any further manipulation, was described and subsequently, having been tested in a number of other collaborating centres, emerged as effective, thereby avoiding the need for any further immunosuppression. Interestingly this is now a standard form of management which, at least in our hands, is associated with a high remission rate at least in acute myeloid leukaemia. Acute and chronic graft-versus-host disease, in traditional sense, are no longer seen. However an interesting form of the unusual immuneologic manifestations occasionally occurs in the form of a skin rash which is regarded as a late onset of the acute variant. A further association is with cytomegaloviral seroconversion but progression to pneumonitis or other organ involvement has largely been prevented by proactive ganciclovir administration.

Audit, accreditation and current status
For an unbroken period of 30 years the same group have reported consecutive cases first to the International and then to the Autologous registries which are now combined as Center for International Blood and Marrow Transplant Research. Latterly accountability is also scrutinised for continued active participating membership of the American National Donor Program. Such peer review is regarded as mandatory to maintain standards and, following audit, accreditation has been uninterrupted for the past three decades.

Scrupulous peer review is maintained

Figure 4: Overall survival in adults
One of the more interesting aspects of this programme is the similarity in survival by Kaplan-Meier analysis between consecutive patients with bone marrow exposed to Campath 1-G in-the-bag, peripheral blood treated with the same immunoglobulin or humanised variant and autografts. This particular approach is notable for the lower level of graft-versus-host disease, high remission rates in acute myeloid leukaemia but with an increasing incidence of cytomegaloviral positivity which is the subject of ongoing investigation.

* Group autograft PBSC
- Group BM 1G
- Group PBSC 1G
- Group PBSC 1M
Extrapolation to restore medicine rationale

Philosophers pose questions in abstract form. Eticists and moralists refine these concepts by debate leaving scientists to provide answers through systematic study. This framework is helpful in understanding how changes in emphasis from the conventional treatments over yesteryear come to be replaced by more proactive or preventive current strategies. Thus, as an example, none would question the use of prostheses to improve the mechanical disability when limbs are lost or the increasing sophistication that makes more user friendly, the use of living tissues is, however, viewed quite differently. Here, although the distinction is somewhat artificial, future advances lie in two broad directions. Firstly there is the controversial area of embryonic tissue and its use in reproductive or therapeutic cloning. It could responsibly be argued that this field needs to advance but to do so in a properly regulated way thereby capitalising on the immense potential benefit in a wide range of congenital and acquired disorders that affect the human race.¹¹ Legitimate reservations extend to how the material is derived and there is a clear need to regulate clinical application.¹² These concerns are not central to the present debate. Secondly, contrasting and of major relevance, as the population ages with subtle changes in immunologic competence and decreasing tissue integrity there is a need for a conceptual shift to supplement physiological replacement where examples include degenerating neural tissue as in Parkinson’s and Alzheimer’s diseases, diabetes, damaged joints and cardiomyocytes.¹³ It is in this context that the immunohaematopoietic stem cell can be examined further looking at the question of plasticity and some preliminary data of its role in selected organ systems.

Plasticity

Careful scrutiny of experiments in nature, exemplified by response to injury, reveals the presence of rests or cellular collections that have the capability of expanding into mature and functional progeny. These are more abundant where turnover is rapid as in the epithelium and blood. Such a belief may be too restrictive since evidence is accumulating that differentiation may occur across lineage boundaries and this phenomenon, described as plasticity, may offer a novel therapeutic strategy to facilitate tissue regeneration.¹⁷
Interpretation of publications need to be tempered by an understanding of technical details that include selection of the study material and the distinction between fusion as opposed to transdifferentiation. Nevertheless the ability to harvest readily accessible adult stem cells may open previously inconceivable treatment options. In view of the ease with which bone marrow can be obtained and evidence of its flexibility in generating myeloid and lymphoid lineages as well as mesenchyme—the latter capable of differentiating into bone, cartilage and fat—highlight the choice of haematopoietic stem cells to examine advances in this field.

**Current or potential applications**

Understanding the way in which the latter population gives rise to blood formation interchangeably with non-haematopoietic lineages challenges the concept of the hierarchical model and has led to speculation that the facts are more compatible with the kinetic concept in which there is a continuum within this compartment. Importantly these emerging concepts sound a word of warning since neoplastic changes may occur in the course of these manipulations.

Two extensive reviews argue the evidence for the presence of organ specific stem cells and possible contributions from marrow stem cells. In reviewing data from mice coupled with limited clinical experience, and recognizing the need, as noted above, to separate fusion from transdifferentiation, a number of areas emerge for specific study and where there is already data available. Prominent among these are skeletal and cardiac muscle, liver, skin, gastrointestinal tract, lung, pancreas, kidney and of particular interest, the central nervous system.

**A concluding perspective**

Conventional bone marrow transplantation has, in the last 40 or 50 years, undergone enormous development and now continues to be translated into great saving of life. Morbidity is being reduced as support and nutritional intervention improve, infective episodes are more effectively treated and graft-versus-host disease skillfully controlled. Immuno-haematopoietic stem cells are routinely recovered from the circulation after mobilisation using apheresis techniques and there is daily wider recourse to umbilical cord blood. In parallel histocompatibility matching is more precise and the role of reduced intensity conditioning extends the age of the recipient and offers an opportunity to test potentially new immunologically mediated benefits exemplified by anti-tumour destruction through supplementary donor lymphocyte infusion. Such momentous advances provide an enormous international database from which to start moving these sophisticated procedures innovatively into the field of restorative medicine. Thus, given that most organs house small populations of stem cells with specific capacity to effect local repair, and nowhere is this better demonstrated than in blood formation, there arises the question of whether such rather narrow concepts should not be reviewed. Leaving aside the more controversial issues of reproductive and therapeutic cloning from embryos and focusing on the adult population with regenerative capacity, the answer would appear to be—yes. Evidence increasingly supports observations that this population has a property described as plasticity to explain their presence during reparative processes in a wide range of many other sites from muscle, through skin and gastrointestinal tract to the central nervous system.

Questions about the interpretation of this phenomenon abound including debates as to whether this is simply cell fusion or, in actual fact, transdifferentiation. Nevertheless it is precisely these important considerations that led to the formation of a South African Study Group that recently held an inaugural workshop to coordinate and support locally based investigators. Understandably one of the discrete working parties, and for good reason, will focus on the long experience of bone marrow transplantation in this country, aiming to develop it as a model to continue the exploration of the potentially important role of these procedures in this new field of restorative medicine.

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Immunohaematopoietic Stem Cell Transplantation-Introduction and 35 Years of Development in South Africa-The Historical and Scientific Perspective

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Bone marrow was the traditional graft source when we introduced these procedures to South Africa. Technical details were established using rabbits as the experimental model with translation into a formally structured clinical programme at the Constantiaberg Medi-Clinic, based in the Groote Schuur Hospital, in 1972. Lack of any infrastructure was overcome by acquisition of the first continuous-flow cell separator in sub-Saharan to provide for granulocyte transfusions. This was shortly followed by creating a dedicated platelet donor panel and establishing a specialised laboratory for clonogenic assays, flow cytometry, programmed freezing and including cryopreservation. Development was constant and seamless but four distinct periods are recognizable. Firstly, guided and constantly encouraged by Professor E Donnall Thomas, was use of an unfractionated mononuclear population derived from multiple sternal and iliac crest aspirations where complications, as in other centres, included rejection and, particularly troublesome, acute as well as chronic graft versus host disease. The second was centred on cyclosporin-A in association with Professor Jean Borel at Sandoz in Basle leading to a decrease in the incidence and severity of the latter immunologic phenomena but not to their abrogation. Thirdly was the opportunity of working with Professor Herman Waldmann and Dr Geoff Hale first in Cambridge and latterly in Oxford on immunosuppression achieved via ex-vivo T-cell depletion within the broad ambit of the Campath users group. Here there was pioneered the alternative new approach of adding the anti-CD 52 monoclonal antibody only to the graft in what has become known as in-the-bag technique. The fourth, securely based on early laboratory and clinical experiences, was a switch to use of peripheral blood stem cells mobilised into the circulation with stimulatory peptides.

In 1995 this original transplant team relocated to a new academic centre in the private sector and has continued to actively refine the programme over the subsequent decade: the facility at Groote Schuur hospital continues independently. Early recognition that accountability for these expensive and high profile procedures was an important obligation led to consecutive transplants being reported to the International and Autologous registries and now continuing to the Centre for Bone Marrow Transplant Research concurrently with the European Bone Marrow Transplant Registry. This disciplined approach has ensured that all data undergoes constant audit and, on such a basis, underpins the unbroken accreditation extending over more than three and a half decades. With difficulties in finding sibling donors a further achievement was creation of The South African Bone Marrow Registry and now a proposal to also start a national transplant registry that will complement the survey currently being conducted, on a worldwide basis, by the European Group for Blood and Marrow Transplantation. It is concluded that a properly constituted and functioning multidisciplinary team can cost-effectively carry out immunohaematopoietic stem cell grafting even in an under-resourced country with outcome approximating that reported from recognized First World reference centres. The caveat is that, outside such comprehensive units, results may be less impressive thereby arguing for resource allocation being directed to academically-designated, rather than incentive-driven, preferred providers.

Summaries of Haematopoietic Stem Cell Transplantation Programs in Countries of Africa, Asia and the Pacific

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Professor E. Donnall Thomas developed the concept and established the technical feasibility of bone marrow transplantation in a canine model. He and his team then courageously pioneered translation of these orderly research studies into the clinic and first proposed what have become the currently accepted indications.\textsuperscript{2} From the start of these activities in Cooperstown to the Public Health Hospital in Seattle and finally to the Fred Hutchinson Cancer Centre he has through sheer talent, inspired leadership and a unique compassion for patients and every member of his staff alike, attracted generations of Fellows who have gone on to sustain the ever clearer definition for usage and unravelling of early and now late side-effects. The hallmark of these graduates is his clear imprint of identifying a clinical problem and then, increasingly, applying cellular and molecular biologic techniques to understanding pathogenesis as a basis for refined or focused intervention. Small wonder that such achievements were recognized by award of the Nobel Prize.

It would be inappropriate to describe the historical parallels that took place in South Africa without the preceding acknowledgement or an explanation of how the programme in sub-Saharan Africa started. During the years as a haematology Fellow in Seattle with Professor Clement A Finch there was regular contact and exposure to the excitement surrounding bone marrow transplantation. While it would be both difficult - and invidious - to select from that group of dedicated investigators more than a representative mention of four that remain currently active including Dr Rainer Storb, Dr Fred Appelbaum, Dr Joachim Deeg and Dr Jean Sanders with whom, among others, we are privileged to maintain association that now extends back over more than 30 years.

One other event that cannot be overlooked was the momentous achievement of Professor Christian Neethling Barnard in carrying out the first human heart transplant in the world. It was into that receptive environment, prevailing at the University of Cape Town and Groote Schuur Hospital, that one had the great honour of being appointed as the Foundation Professor of Haematology. The inevitable question from Sir Richard Luyt and the appointments committee was what direction the new department had selected for particular focus! With no in-depth training in immunology and precious little in transplantation biology it was with tenuity that this emerging area of interest-bone marrow grafting-was chosen. And so the die was cast. The brief historical record that follows freely acknowledges unrestricted encouragement from innumerable colleagues of all persuasions, particularly during the early developmental with periods of frustration balanced by elation, and the many members of the multidisciplinary team who, collectively, made it a reality. It equally reminds us all never to lose sight of that extraordinary courage shown by patients and their families alike.

From individual centre and case studies to the meticulously orchestrated collection and analysis of data by the Centre for International Bone Marrow Transplant Research, indications in adults and children are now clearly defined (Figure 1)\textsuperscript{3} while outcome remains the subject of continuous review and updating.\textsuperscript{4} Against this background a number of relevant observations emerge that logically start with patient selection and consideration of variables including age and comorbidity. It is here that collective experience of the multidisciplinary team is crucial with best results reflecting an under-appreciated centre effect.\textsuperscript{5}

All important is the conditioning of the recipient to accept the incoming graft but, in selected instances and depending on diagnosis, may be the need for further chemotherapy directed at eradicating residual disease. Historically myeloablative preparation with high-dose chemotherapy\textsuperscript{6} or irradiation\textsuperscript{7} and more innovatively by use of radioconjugates\textsuperscript{8} continue to undergo study. An area of particular interest has been the use of anti-CD 52 monoclonal antibodies which can be given either to patient or a used ex vivo in what has become known as 'in-the-bag technique'.\textsuperscript{9} Recognizing the morbidity and mortality associated with this step has resulted in description of reduced intensity regimens that make it possible to extend the upper age limit but this may be offset by higher disease relapse rates. However reversal of the latter complication is possible in some cases by capitalising on antimicrobial effects mediated by alloreactive T-cells in the form of delayed donor lymphocyte infusion.\textsuperscript{10} At this point remains the unresolved and challenging issue of optimum post-transplant immunosuppression ranging from traditional corticosteroids with cyclosporin and methotrexate through a wide range of new and potentially more effective-possibly even safer-options.\textsuperscript{11}

This sophisticated form of treatment is arguably best carried out in a dedicated reverse isolation unit or protected environment but debate rages on precisely what defines such a physical facility or plant. Arguably, of far greater importance, is investment in a properly constituted cross-disciplinary management team with, at least in Africa, input from practitioners of alternative or complementary medicine. Also there is a need for reliable and safe supply of blood and related products, a laboratory capable and registered as competent to carry out apheresis procedures, experienced in programmed freezing and licensed for cryopreservation in terms of locally promulgated acts for dealing with human tissues.\textsuperscript{12} Donor availability, particularly with increasing use of matched unrelated volunteers and alternative sources including cord blood, are those unique considerations securing access to tissue typing with...
immunogenetics accreditation. Transplants not carried out in such an organised, audited and accredited centre, designated as competent for both harvesting and grafting, are viewed as inappropriate and to be discouraged.

Then, in the short term defined as the first hundred days, is transplant related mortality which has a number of contributors. These include adequacy of high-resolution histocompatibility matching between donor and recipient.

Secondly quality of the graft defined in a number of different ways ranging from mononuclear cells to CD 34 expressing population or repopulating potential documented in clonogenic assays as a long-term colony initiating cells. Thirdly the ever-improving supportive care extending from the isolation facility to regular participation of consultants in infectious disease, cardiology and pulmonology coupled with the all-important and dedicated nurses and other para-medical professionals.

Integral to this period, but extending throughout long-term care, is attention to quality-of-life that requires specific documentation and typically would involve psychosocial counselling, psychology and liaison psychiatry. As volume, and therefore referrals increase, emerges a new aspect in which patients increasingly accept responsibility for their care and work closely with community medical and nursing staff. The sensitivity and considerable thought, as well as an extraordinary amount of communication so vital to achieving success of this exercise, directly impacts on continued support and acceptance of the role that the transplant centre plays in an entire region. In the reverse direction is the need for an effective data-capture system vital to make possible correlation of all events with survival. Unless well orchestrated such limitations assume disproportionate magnitude in under-resourced countries and place new and particular onus on those willing to accept responsibility for carrying out these procedures in having to maintain scrupulous follow up and schedule specialised investigations at clinic visits (Figure 2).

The cardinal responsibility of transplant teams is to maintain accountability and transparency defined by regular audit on which will be based accreditation of status as transplant and harvest centres. This requires reporting of consecutive patients to international registries with matching records of activity via national surveys. And, additionally, the constant analysis and publication of results. Only in this way can there be any confidence that these hazardous and high cost procedures, increasingly being carried out in the Third World, approximate standards of care using international norms. Arguments that such criteria do not apply to developing countries, where less good outcome might be conditioned, is clearly specious, as well as inappropriate! Such an attitude should not be entertained and much less supported—particularly since there is evidence that precisely the opposite is true.

It is against these observations that first the introduction and then continuous development of immunohaematopoetic stem cell transplantation in South Africa over a 35-year period can now be detailed. There emerges the conclusion that, at the present time, they continue to provide life saving and cost-effective, as well as the resource-appropriate therapeutic options in selected.
Formal outcome analysis. Implicit in such a rather more constructive endeavour will be the need to comply with ethics and research rules including attention to issues that include donor confidentiality. This should be easy given the simple expedient of only completing already available, and circulated, relatively standardised data capture sheets.

**ESTABLISHMENT OF THE CAPE TOWN INFRASTRUCTURE**

When this programme was inaugurated in 1970 blood was provided in glass bottles and platelets available only as single unit concentrates. To ensure that adequate transfusion support would be available an IBM 2990 was secured by donation and this prompt action initiated a long and ongoing collaboration with Professor Jeane Porter Hester (Figure 5). Although initially used for granulocyte transfusion the apheresis technology rapidly became the anchor, together with creation of a dedicated volunteer panel for donors, as one of the crucial moves towards realising clinical transplantation. Physical facilities were limited and the start-up procedures were carried out with literally unlimited encouragement in the newly built block for cardiac transplantation through the courtesy of Professor Christian Neethling Barnard. As a result of his programme histocompatibility testing also became available having only recently been introduced into our country. It was therefore possible to get class I by serology and some idea of class II by mixed lymphocyte reaction. These shortcomings were relatively soon appreciated and, in close collaboration with Professor Emette du Toit, high-resolution typing became available with eventual immunogenetic testing approved in her laboratory. The activity of the South African Bone Marrow Registry is reflected in the latest statistics report (Figure 6). The technical procedures of marrow processing including characterisation of the recovered mononuclear product necessitated development of a rabbit model in association with Professor Bruno Speck and Professor Alois Gratwohl in Basle to refine the methods and almost immediate establishment of flow cytometry and clonogenic assays using these transplants between strains showed that acute and chronic graft-versus-host disease closely paralleled what was being seen in the clinical programme. These manifestations were used to document the effects of cyclosporine-A just made available by Professor Jean Borel at Sandoz in Switzerland. The same approach proved crucial in evaluating the administration of the Campath series of monoclonal antibodies to the recipient but subsequently showing that even more effective immunosuppression was possible by exposing the graft to the protein ex vivo in what has subsequently been described as in-the-bag technique. This method has emerged as surprisingly rugged in abrogating classical acute graft-versus-host disease.

**DEMOGRAPHIC CONSIDERATIONS AND CURRENT TRANSPLANT ACTIVITY**

South Africa has a surface area of 1 219, 08 km² (Figure 3) with a population of 47 849 800 and density (135/km²). Complete records are available from the time bone marrow transplantation was started by the originally designated team (Figure 4). Now, with the recent proposal that a national database be started, matching activities throughout the country can be collected hopefully via the existing Bone Marrow Registry. Such voluntary participation should, in future, make these activities a matter of general record. At the same time South Africa has been included in a survey by questionnaire circulated from the European Bone Marrow Transplant Registry gathering essentially the same figures. Clearly these two activities need to be complimentary. Logically this exercise is but an initiating step which can easily be extended to properly managed centres that enjoy international designation.
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<td></td>
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<td>1998</td>
<td>70</td>
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<td>Bloemfontein Academic Hospital</td>
<td>Vernon Louw and Hymne Louw</td>
<td>Not yet activated</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>Durban</td>
<td>Vinod Jogessar and Natasha Sewpersad</td>
<td>2007</td>
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<td>Albert Luthuli Academic Hospital</td>
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<td>Pretoria</td>
<td>Jackie Thompson and Hannelie Duvenage</td>
<td>2006</td>
<td>44</td>
<td>12</td>
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<td>**Mary Potter Private Oncology Centre</td>
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*Adam Nosworthy, Devon Woodley and Georgia Demetriou - Joint Witwatersand University and Donald Gordon Medical Centre
**Graham Cohen, Lydia Drosti, Ananda Korsor

Receipt of these figures from each participating centre is acknowledged with thanks to the Principal Investigator and Reporting co-ordinator.

Figure 5. The Apheresis Unit [Seen here is Professor Jeanie Hester Porter with the latest generation Cobe Spectra cell separator and staff members].
Figure 6. South African Bone Marrow Registry Quarterly Statistics.

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Number of Registered Donors:</td>
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<td>117#</td>
<td>62 293</td>
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<tr>
<td>HLA-AB typed</td>
<td>58 470</td>
<td>58 518</td>
<td></td>
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<tr>
<td>HLA-ABDR typed</td>
<td>3 706</td>
<td>3 725</td>
<td></td>
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<tr>
<td>% HLA-ABDR typed</td>
<td>5.9%</td>
<td>6.0%</td>
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<td>Searches for Donors</td>
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<td></td>
<td></td>
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<tr>
<td>- Preliminary Searches</td>
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<td></td>
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<tr>
<td>Local Haematologists</td>
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<td>70</td>
<td>2 502</td>
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<td>International Registries</td>
<td>490</td>
<td>20</td>
<td>510</td>
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<tr>
<td>- Activated Searches South African Patients</td>
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<tr>
<td>New activated searches this quarter</td>
<td>1942</td>
<td>50</td>
<td>1992</td>
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<tr>
<td>All active searches this quarter*</td>
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<tr>
<td>South African Patients receiving matched unrelated transplants**:</td>
<td>109</td>
<td>4</td>
<td>113</td>
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<tr>
<td>Local Donor</td>
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<tr>
<td>International Donor</td>
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<td>4</td>
<td>83</td>
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<tr>
<td>% Local Donor</td>
<td>27.5%</td>
<td>26.5%</td>
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<tr>
<td>Peripheral Blood Stem Cell Donations by South African Donors:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Local Recipients</td>
<td>37</td>
<td>0</td>
<td>37</td>
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<tr>
<td>International Recipients</td>
<td>30</td>
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<td>30</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0</td>
<td>7</td>
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</tbody>
</table>

* At open patient files, including patients for whom a donor has been found. # Net gain for the period but who have not yet been transplanted. Note: 464 donations this quarter.

**First 465 donors. With thanks to Professor Enette De Toit, Mrs Tanya Schutte and Mrs Veronica Borelli for permission to use this data.

and so substantially diminishing, almost to the point of extinction, the subsequently occurring chronic variant.21

THE CLINICAL PROGRAMME

Constant development, in a structured and actively evolving series of experimental and clinical studies, fell into four convenient but artificially distinct periods that characterise these activities in sub-Saharan Africa and particularly as chronicled at the University of Cape Town and Groote Schuur Hospital.16

UNFRACTIONATED BONE MARROW ERA mirrored experience occurring elsewhere in the world. Engraftment was often successful but plagued by graft failure and substantial morbidity as well as significant mortality consequent upon the syndrome of acute graft versus host disease (Figure 7). Here often-profound injury to skin, biliary endothelium and enterocytes, compounded by severe infection, responded only partially to immunosuppression with corticosteroids and methotrexate24 with extension to a severe sclerosing chronic form that created intractable disability in adults and children alike (Figure 8).25 In addition to the severe disability caused to recipients with devastating disorganisation of family life there emerged a more profound and often underplayed psychological injury to medical and nursing staff alike. This caused high staff turnover and understandable criticism from detractors of transplantation in general and particularly to the use in haematology with the focus on the perceived worse side effects in children.

CYCLOSPORIN-A ERA started when this undecapeptide was isolated by Jean Borel in Sandoz and our involvement in some of the earliest investigations of this biological breakthrough that was again thoroughly explored in the rabbit26 (Figure 9). Here, as when the experimental studies were translated into the clinic, incidence and severity particularly of acute graft-versus-host disease, was decreased but the broad range of complications changed surprisingly little. Nevertheless this was a seminal step in
Figure 7. Acute graft-versus-host disease. The syndrome of extensive skin desquamation, jaundice and large volume diarrhoea had a sobering effect in the initiation phase of the programme.

Figure 8. Chronic graft-versus-host disease. While many recipients survived a percentage had severe sclerosing variant of this complication of allografting with unmanipulated bone marrow.

Figure 9. Rabbit as experimental model. The early technical development was carried out using inbred strains allowing controlled allograft dosing with variation in severity of acute and chronic graft-versus-host disease. This reference was the basis for introduction of cyclosporin and Campath monoclonal antibodies.
being able to reverse weakening confidence of the local medical community in the role of immunohaematopoietic stem cell transplantation and set the stage for the continued active exploration of new and more effective agents.26

MONOCLONAL ANTIBODY OR CAMPATH ERA was in many ways the single biggest advance with which we were directly involved. Thus the opportunity was afforded us of working with Professor Herman Waldmann and Dr Geoff Hale, initially in Cambridge and subsequently in Oxford, through participation in the scientific deliberations of the Campath users group. There was the innovative approach of employing selectively synthesised series of immunoglobulins for the alternative approach of effecting immunosuppression by means of relatively selective T-cell depletion. Experience in the animal transplant model initially, with the lytic IgM protein, showed graft-versus-host disease to be beneficially affected and this could be duplicated in human allograft recipients with improvement in outcome. These events were enhanced first by the use of the opsonic IgG and then, more recently, the humanised chimeric protein confirmed in other countries.27

At about the midpoint of these collaborative studies innovative experimental evidence in our laboratory demonstrated that addition of the immunoglobulin to the graft, prior to infusion alone and without any subsequent immunosuppression using antibody or drugs, impacted favourably on acute graft-versus-host disease. This syndrome virtually disappeared although a late-presenting mild acute variant was recognized limited to skin, being grade 1 and typically responding to topical steroids but seldom needing systemic administration. This was the birth of the in-vitro, ex-vivo or, as it has subsequently become known, Campath in-the-bag technique.28

Overlapping with these developments was the recognition that a stem cell population having many characteristics similar to those derived from the bone marrow could be obtained by the much simpler expedient of recovery from the peripheral circulation usingapheresis technology with enhancement resulting from donor receiving stimulatory peptides in the form of G-CSF.29 A new observation emerged in that cytomegaloviral infections, which had not been particularly prominent whilst using marrow, now increased requiring constant monitoring and prompt administration of gancyclovir to avoid progression from viraemia to the much more hazardous situation of clinical disease typically in lung or gastrointestinal tract.

THE CURRENT ERA, spanning the last 12 years, has been particularly illuminating in the context of an under-resourced area where state support for teaching hospitals continues to relentlessly erode so that commercial ventures have become more receptive to accepting tertiary-level

Figure 10. Overall survival in children. Kaplan-Meier analysis shows that at 6.8 years there is a stable plateau consistent with cure. It is notable that results are similar for idiopathic aplasia, Fanconi anaemia and acute myeloid leukaemia with non-significant differences for lymphoblastic leukaemia and the remaining cases. There is also no difference in outcome for graft source, but a slight benefit for the female gender, which did not attain statistical significance.

Figure 11. Overall survival in adults. One of the more interesting aspects of this programme is the similarity in survival by Kaplan-Meier analysis between consecutive patients with bone marrow exposed to Campath 1-G in-the-bag, peripheral blood treated with the same immunoglobulin or humanised variant and autograft. This particular approach is notable for the lower level of graft-versus-host disease, high remission rates in acute myeloid leukaemia but with an increasing incidence of cytomegaloviral positivity that is the subject of ongoing investigation.
Figure 12. Cost estimate for stem cell grafting in South Africa.

<table>
<thead>
<tr>
<th>Description</th>
<th>Rand</th>
<th>Dollar</th>
<th>Euro</th>
<th>Pound</th>
</tr>
</thead>
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<tr>
<td>Peripheral blood stem cell collection</td>
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<td>3376.89</td>
<td>2308.00</td>
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<tr>
<td>Physician’s fees - daily management</td>
<td>60 000</td>
<td>8104.53</td>
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<tr>
<td>Ward fees</td>
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<td>10806.00</td>
<td>8025.60</td>
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<td>Chemotherapy Conditioning</td>
<td>40 000</td>
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<tr>
<td>Projected Antimicrobial usage</td>
<td>20 000</td>
<td>2701.51</td>
<td>2006.40</td>
<td>1361.92</td>
</tr>
<tr>
<td>Other pharmaceuticals</td>
<td>20 000</td>
<td>2701.51</td>
<td>2006.40</td>
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<tr>
<td>Consumables</td>
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<td>Radiotherapy Conditioning, etc</td>
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<td>7466.89</td>
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<tr>
<td>Placement of Central Venous Catheter</td>
<td>6 500</td>
<td>877.99</td>
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<td>Bone Marrow Aspiration</td>
<td>5 000</td>
<td>675.37</td>
<td>501.60</td>
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<td>Blood and Related Products</td>
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<tr>
<td>Pathologists Fees</td>
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<td>2006.40</td>
<td>1361.92</td>
</tr>
<tr>
<td>Radiologists Fees</td>
<td>15 000</td>
<td>2026.13</td>
<td>1504.80</td>
<td>1021.44</td>
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<td><strong>TOTAL</strong></td>
<td>408 779.40</td>
<td>55 216.03</td>
<td>41 008.75</td>
<td>27 856.16</td>
</tr>
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</table>

responsibilities. Thus it has been possible to relocate, within a single Facility, most of the original team into a privately based Academic Department of Haematology and Bone Marrow Transplant Unit that incorporates the Searl Research Laboratory for Cellular and Molecular Biology. The experiment has been successful proving that it is realistic to maintain the consecutive patient reporting system first to the International and then Autologous registries that are currently combined into the Centre for Bone Marrow Transplant Research. This period demonstrates feasibility of meeting the audit criteria for continued accreditation both as a transplant and harvest centre now extending, for an unbroken period in excess of three decades, as well as accommodating the need for matched unrelated volunteer donors. The latter sparked the need to start a donor registry that has become a national entity providing tissue-typing services for other centres in the country, regularly interacting with corresponding facilities and participates in searches from elsewhere in the world with activity recently updated. The standard is such that endorsement is maintained from the American National Donor Programme as well as the European Bone Marrow Transplant Registry. In parallel outcome has been analysed and reported in both children and adults (Figure 11). While particular relevance of these procedures in patients with lymphoma continues to attract our ongoing and special attention.

An important consideration in the Third World is to balance the need for these high cost procedures against resources available in state hospitals having limited budget and a competing private sector where restraints are of a slightly different nature dedicated by the particular insurance plan available through managed health care. In order that some idea of national activity can be gauged it was recently proposed that a bone marrow transplant registry be established within the ambit of the existing national body being the South African Bone Marrow Registry. The argument in favour of such a base is that it would allow comprehensive or inclusive recording of autologous and all forms of allogeneic transplantation and include alternative sources such as cord blood and matched unrelated volunteer donors and do so in a non-partisan way. It would also permit government, through the Department of Health, access to reliable statistics as to activity of the teams whether they be in university hospitals or private sectors. Additionally it would anticipate future needs for appropriate regulation that may be promulgated in terms of the human tissues act. Here particular issues relate to harvesting, any form of processing or manipulation of grafts and subsequent cryopreservation whilst monitoring movement of these human products not only within the country but a cross-border between countries through the medium of collaborating international registries.

CONCLUSION

The basis for this national experience can be factually recorded in three phases of past achievement and pres-
ent status with a projection for the future. Historically the experimental haematology and subsequent systematic translation into the clinical programme documents the capability of an under-resourced country to provide advanced and life-saving interventions as dictated only by the genuine needs within the community, across the age spectrum and catered for by the state or private sector even where costs are a major consideration (Figure 12). It is equally clearly demonstrated that in those few facilities that have elected to meet the criteria for ongoing audit and accreditation from international peer designations is possible as donor and harvest centre leading to participation in a worldwide community linked through registries that share search and provision of matched unrelated volunteers when these are not available locally. Finally it is time to survey activity within our borders and to this end reporting of consecutive procedures by each practice has been proposed forming a registry to operate within the ambit of the nation’s nationally launched South African bone marrow Registry. The latter initiative is given sharp focus by a similar approach already launched by the European bone marrow transplant Registry and both should be mutually complimentary. Such a proactive move would have the capacity for expansion to document outcome although this would require appropriate ethics and research monitoring. A further attraction is creation of a reliable database providing comparison between different regions of the world and, in local context, a basis to guide and educate practices in terms of regulation for transfusion practices and handling human tissues. The natural end-point becomes academic recognition for properly accredited programmes to safeguard future scientific and training requirements whilst simultaneously reducing any need for commercial or incentive-driven providers.

REFERENCES

Human T-Cell Leukemia Virus (HTLV-I) Antibodies in Africa

Human T-Cell Leukemia Virus (HTLV-I) Antibodies in Africa

Abstract. Antibodies specific for human T-cell leukemia-lymphoma virus type I (HTLV-I) were demonstrated in serum samples from various groups of people in South Africa, Uganda, Ghana, Nigeria, Tunisia, and Egypt. The samples had been collected for other purposes and were presumably selected without bias toward clinical conditions associated with HTLV infections. Regional differences in antibody positivity were observed, indicating widely distributed loci of occurrence of HTLV on the African continent in people of both black and white ancestry. Two patients with high titers of antibody to HTLV-I had some signs of adult T-cell leukemia-lymphoma. In several groups a high frequency of false positive serum reactions was indicated when specific confirmation steps were included in the assay. Further characterization of these sera revealed highly elevated immunoglobulin levels, possibly due to polyclonal activation of immunoglobulin synthesis in these subjects. The possibility that related cross-reactive human retroviruses coexist in the same groups was not eliminated.

The human T-cell leukemia-lymphoma viruses (HTLV) are a family of related retroviruses originally isolated in the United States from patients with T-cell lymphoma and cutaneous manifestations (1). A particular subgroup of the family, HTLV type I, is linked to the cause of these malignancies, which share clinical and epidemiologic features with the disease called adult T-cell leukemia-lymphoma (ATL) that occurs in certain regions of Japan (2, 3) and in persons of African ancestry in the Caribbean Basin (4) and in the southeastern United States (5). An atypical chronic lymphocytic leukemia in Nigeria is also suggestive of an association with HTLV (6), as is the high incidence of antibodies cross-reactive with ATL in Old World primates captured in Kenya and Ethiopia and housed in West Germany (7) and the United States and Russia (8). Although the mechanism of transmission of HTLV is currently unknown, horizontal transmission is clearly implicated by molecular and epidemiologic analyses (9, 10). HTLV seropositivity in regions endemic for ATL is elevated overall in the general population and further elevated among close family members of cases and in recipients of blood transfusions (11, 12).

The present study, which is mainly descriptive, was undertaken to investigate the occurrence of antibodies to HTLV-I in various groups of people in widely distributed areas of the African continent. We studied serum samples that had been collected for surveys of diseases with no known association with HTLV-I and samples from hospital-based clinic patients. We used a highly sensitive enzyme-linked immunosorbent assay (ELISA) to detect antibodies to HTLV-I (13) (see Table 1). Because of the diversity of the test groups, our data cannot be used to make strict epidemiological comparisons, but can be used as a means to compare the distribution of virus antibody positivity with previously reported studies of exposure to the virus (2, 12, 14) in similar or analogous groups.

The testing procedure was performed in two steps (legend to Table 1). In step 1, all samples were screened to determine quantitative levels of antibody binding to HTLV-I. In step 2, "candidate" positive sera were selected and tested for specificity in one or more confirmatory steps. The screen-test results are expressed as a ratio (R) to a standard reference normal serum to control daily variations in test results (13). The threshold level, R = 2, was not expected to exclude negative sera. The use of this cutoff for confirmation was based on prior experience with normal U.S. blood donors where samples with a screening ratio of <2 are negative in the confirmation assay. This reference normal serum level and the threshold for detection of sera confirmed as being positive for antibody to HTLV-I were derived from an analysis of 1210 U.S. blood donors (15).

Specificity was considered confirmed when sera passed either one of the confirmatory tests described in Table 1. The accuracy and precision of the antibody-blocking procedure was verified by measuring the fractional reduction of antibody binding for mixtures containing a predetermined ratio of HTLV-positive antibodies to the reference normal serum. The results plotted in Fig. 1 show excellent agreement with the predicted results at low levels of positive antibody and deviation within acceptable limits due to incomplete blockade at the higher levels of human antibody. Of those sera failing confirmation by antibody blocking, only three were confirmed by absorption with virus-positive cells.

The values for the numbers of sera from the groups exceeding the screen-test threshold and for the numbers of confirmed positive sera in each of these groups are presented in Table 1. The median values for screen ratio (R) within the groups were in most cases close to the median value of 1.77 found for U.S. donors (13). However, median values of R for samples from Tunisia and Ghana were two to three times higher. This reflected the absence of a simple correlation between the prevalence of confirmed positive sera and the proportion of sera exceeding the screen threshold level (R = 2); for example, the proportion of confirmed positive donors in the Ugandan group (21 percent, which was highest of all groups) was two times higher, while the proportion of samples exceeding R = 2 was only one-half that of the Ghanaian groups.

We investigated some of the reasons for this apparent high rate of false positivity. Among the Ghanaian samples, 28 out of 67 with high ratios (R = 6) were nonconfirmed. The mean and median immunoglobulin G (IgG) levels of these 28 samples were, respectively, 130 and 106 mg/ml compared to 9 mg/ml for the standard control serum (measured by ELISA with immunopurified goat antise-
Table 1. Distribution of HTLV-I antibody among African donors. An indirect ELISA microtest to detect serum antibodies was used (13). Briefly, HTLV-I was purified by rate-zonal ultracentrifugation, disrupted, and coated into the wells of microtiter plates. Portions (3 μl) of test sera, control sera, and normal human sera were incubated overnight at 4°C in wells containing 100 μl of 20 percent heat-inactivated normal goat serum and were washed with PBS. After reaction with peroxidase-labeled goat antiserum to human IgG, sera with absorbance values two times greater than the normal control level were considered positive by a colorimetric assay, which involved the addition of phenylmethylsulfonyl fluoride (40 μl) to each well, followed by incubation for 30 minutes at 37°C. The absorbance at 630 nm was measured with a microtiter plate reader. Positive sera were confirmed by retesting the same lot of sera at a 1:2 dilution and with a titer of 100,000 or more. The sheep antiserum used in these tests required absorption with one volume of serum to each serum to be tested and with three volumes of serum to each serum to be tested and with three volumes of serum to each serum to be tested and with three volumes of serum to each serum to be tested. The antibody was determined by a 1:20 dilution of the serum samples. The ratio of optical density (O.D.) of the sample to the negative control was compared with a standard curve.

<table>
<thead>
<tr>
<th>Geographic and racial background of donors</th>
<th>Group characteristics</th>
<th>Number tested</th>
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<th>Number with R ≥ 2</th>
<th>Number positive</th>
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<td>Normal comparison population</td>
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<td>19</td>
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<td>Burkitt's patients and normal comparison population</td>
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<td>1.71</td>
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<td>18</td>
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<td>104</td>
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</tbody>
</table>

* Sera from Egypt were obtained from infected disease patients, National Research Unit No. 3, Cairo, Egypt. † Sera from Tunisia were obtained from healthy patients with no apparent disease, Tunis, Tunisia. § Sera from Ghana were obtained from patients with adult T-cell lymphoma. § Sera from Uganda were obtained from patients with adult T-cell lymphoma. ¶ Sera from Nigeria were obtained from patients with adult T-cell lymphoma. # Sera from South Africa were obtained from patients with adult T-cell lymphoma.

The positive sera were further analyzed by a nested-PCR assay for the presence of the HTLV-I proviral DNA. The positive sera were then subjected to a confirmatory test, and the results were compared with the results of the initial test. The results of the confirmatory test were then compared with the results of the initial test to determine the accuracy of the initial test. The data were analyzed using the chi-square test, and the p-values were determined using a significance level of 0.05. The results showed that the initial test was highly accurate, with a sensitivity of 98% and a specificity of 90%. The data also showed that the confirmatory test was more accurate than the initial test, with a sensitivity of 100% and a specificity of 95%.
years) compared to endemic populations in Japan where the antibody positive rate for the equivalent age group is approximately 5 percent (14).

The highest rate of antibody positivity, 21 percent, was found in a serum collection from Uganda (Table 1). The sera had been collected from patients with Burkitt’s lymphoma and from normal comparison groups in the West Nile region of Uganda over the years 1970 to 1972 as described previously (18). Since we were unable to discern any difference in the positivity rates between these groups they are listed together in Tables 1 and 2.

In a survey of patients with T-cell leukemia/lymphoma diagnosed in Nigeria (19), two cases fitting the typical characteristics of HTLV-associated disease were identified. The first case, a 19-year-old male student from Lagos, had an aggressive T-cell lymphoma with a high count of white blood cells that included cells of pleomorphic morphology, cutaneous involvement, and hypercalcemia. The second, a 57-year-old woman, had a clinically aggressive leukemia/lymphoma with generalized adenopathy and visceral involvement. She died shortly after diagnosis. Sera from both patients contained a high titer of antibodies to HTLV-I (see Table 2). One additional patient with ATL with high HTLV antibody titer, a native of Zaire, has been observed in Paris (20).

In Cape Town, 5 to 10 percent of patients with various malignant diseases had HTLV antibody (Table 1). There were no reported cases of ATL, and the greatest number of HTLV-I antibody-positive cases occurred among the myeloid malignancies (10 percent), although one patient with T-cell leukemia was positive. In areas of southwestern Japan that are endemic for ATL, the frequency of HTLV among patients with myeloid leukemias was 16 percent (2). Our present results with regard to the distribution of HTLV-I antibody within disease categories agree very well with the pattern found in the Kanto district, a nonendemic area of Japan (12). In that district, two important factors contributed to the high rate of HTLV-I antibody positivity in patients with diseases not linked with this virus. One was that the patient population largely originated from an endemic area and the other was the frequent use of blood transfusions in the management of myeloid leukemias (12).

With one exception, serum samples positive for HTLV antibodies were found in groups from all of the African subcontinental regions tested: Tunisia; Ghana; Nigeria; Uganda; Cape Town, South Africa; and Egypt. The absence of HTLV-I antibody in sera from the Johannesburg group may be related only to sample size and probably indicates a lower prevalence than in the other African groups. Race did not appear to be a disposing factor since positive serum samples from Cape Town were mainly of white origin. Although antibody-positive samples were detected in the Tunisian (taken as a whole) and Egyptian groups, the combined factors of frequency and titer found (Tables 1 and 2) were not significantly higher than the baseline for normal donors in the United States (15, 21). Both of the positive samples from Tunisian patients with lymphomas had very low titers, that is, 30 and 37, and the lymphomas were of B-cell origin. For reference purposes, among normal blood donors determined by comparable techniques, the HTLV-I antibody positivity rates between nonendemic and endemic regions of Japan range from 2 to 12 percent (2) and in the United States, 0.9 to 2.8 percent (15, 21).

The typical HTLV-I-associated disease as it occurs endemically in Japan, the Caribbean Basin, and sporadically elsewhere (22) is characterized by the occurrence of malignant cells of varying size and pleomorphic morphology with deformed nuclei and mature T-cell surface marker phenotype. It is often characterized by its onset at a relatively young age; by its aggressive clinical course with poor prognosis; and by the enlargement of lymph nodes, spleen, or liver; elevation of white blood cell count; hypercalcemia; and occasional skin involvement. Our data reveal increased levels of HTLV-I-specific antibodies in diverse African groups (compare with (23)) and suggest that the antibody levels in regions of South Africa, Ghana, Nigeria, and Uganda equal or exceed those found in previously described areas where HTLV-I and ATL coexist. It

Fig. 1. Correlation between specific antibody binding and its susceptibility to competition by HTLV-specific antiserum. A dilution series containing varying ratios of serum positive for HTLV-I antibodies to serum negative for antibodies was constructed by diluting a positive serum (serum F4608; titer = 3000) with our standard negative serum (serum F4600). Each dilution and the negative control serum were tested for HTLV-binding in wells pretreated with unlabeled sheep antiserum to HTLV-I and normal sheep serum as described in Table 1. The ELISA absorbances at 490 nm were measured and expressed as the ratio to negative human serum treated with normal sheep serum (abscissa); and the relative reduction of each diluted positive human serum after incubation with sheep antiserum to HTLV-I (ordinate). Solid line: a theoretical curve was constructed on the basis of the expected relationship between the fractional content of specific antibodies (abscissa); broken line: the maximum achievable reduction (ordinate).

<table>
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would be interesting to conduct systematic surveys of patients with adult non-Hodgkin's lymphoma in various regions of the African continent, with an emphasis on clinical, pathologic, and immunopathologic features of the disease. A study of sera from African patients with known or suspected T-cell malignancies, including the acquired immune deficiency syndrome (AIDS) (24), would help to clarify the distribution of the HTLV family and the diseases associated with it, especially in view of the high HTLV-I antibody level in the Ugandan group, the occurrence of AIDS in neighboring Zaire (25), and the occurrence of Kaposi's sarcoma along the equatorial region of Africa with its highest prevalence in eastern Zaire and western Uganda (26).

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References and Notes


15. In a study of 178 normal U.S. blood donors from Burlington, Vt.; Birmingham, Ala.; and Houston, Tex., the rates of positive HTLV-I antibody-positive sera were 0.9, 2.1, and 2.8 percent, respectively. The range of titers was 30 to 330. The median value of R for all groups was 1,17, and 5 percent of sera with R > 2 were positive [W. C. Saxinger et al., in preparation].

16. We expect that these and other types of indirect assays currently applied without a specific confirmatory step, including immunofluorescence assays that rely on the use of live or fixed HTLV-I-infected lymphocytes, would also be sensitive to such drastic elevations in IgG and could lead to overestimation of seropositivity in similar cases.


20. Patient was originally seen at the Assistance Publique-Hopitaux de Paris (G. Lellouch and M. Robert-Guroff, personal communication).


24. R. C. Gallo et al., Science 228, 865 (1985); F. Barbi-Sinussi et al., ibid., p. 856; M. Ezers et al. et al., ibid., p. 829; R. C. Gallo et al., ibid., 224, 500 (1984).


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AIDS defining lymphomas in the era of highly active antiretroviral therapy (HAART) – An African perspective

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Abstract

The intermediate to high grade B-cell non-Hodgkin lymphomas are now one of three malignant AIDS defining conditions. The others being Kaposi’s sarcoma and cervical carcinoma. While co-infection with oncogenic agents including the human herpes 8 or Epstein-Barr virus offer targets in preventive treatment strategies for these AIDS defining lymphomas (ADL), administration of highly active antiretroviral therapy leading to immune reconstitution permits use of standard or even high-dose cytotoxic drug regimens with curative intent. It is not certain whether this should be done concomitantly or sequentially. Additional benefit may derive from infusional or high-dose chemotherapy regimens depending on the histological subtype while use of monoclonal antibodies such as rituximab or immunohaematopoietic stem cell transplantation needs to be further evaluated within controlled studies. Socio-economic considerations have an impact especially in resource limited settings while availability of tools for appropriate geno-phenotypic diagnosis and immunological monitoring such as the CD4 cell count will play an important role in the risk stratification as well as disease management. While it is generally accepted that the impact of HAART has an overall benefit both in incidence and treatment outcome in ADL, the expanded access to HAART programs are falling short of all targets in Africa. Accordingly focus is given to some of these controversies, including epidemiology, pathogenesis, clinical features, therapeutic options and ethical considerations.

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1. Introduction

The acquired immunodeficiency syndrome has reached pandemic proportions where, in many parts of sub-Saharan Africa, shortened mean life expec-

tancy in adults between 10 and 15 years [1]. This excess mortality is attributable to opportunistic infections and neoplasms, where Kaposi’s sarcoma and primary central nervous system lymphoma emerged as defining conditions 20 years ago followed subsequently by non-Hodgkin lymphoma and carcinoma of the uterine cervix [2]. Retrovirally infected patients have an increased risk of developing Hodgkin variants, myeloma, leukaemia, invasive anal carcinoma and lung cancer but correlation with progressive immunosuppression remains to be established [3].
Kaposi's sarcoma is the subject of a separate report [4]. Of the non-Hodgkin lymphomas, the small non cleaved-cell or Burkitt's type, diffuse large B cell group including immunoblastic, primary central nervous system lymphoma, plasmablastic of the gastrointestinal tract and primary effusion lymphomas are the most common subtypes seen in HIV disease with recognised demographic variations [5].

2. Aids defining lymphomas (ADL)

2.1. Epidemiology, risk factors and impact of HAART

The clinical characteristics of these lymphoproliferative disorders is markedly altered in the presence of human immunodeficiency virus (HIV) infections by the more frequent occurrence of constitutional symptoms and extranodal disease in the bone marrow, body cavities, jaw or rectum [6]. Additionally is the frequent association with Epstein-Barr virus (EBV) or human herpes virus-8 (HHV-8) [7,8] where there is some correlation with the degree of immunosuppression and presence of the primary central nervous system lymphoma subtype. The relative risk of developing one or other of the ADL is between 100 and 600 fold higher than the general uninfected population in contrast to the much lower occurrence of Hodgkin lymphoma [3].

Since highly active antiretroviral therapy (HAART) became the standard-of-care the overall occurrence rate of lymphoma has consistently declined with the evidence that not all benefit equally from these agents. A meta-analysis of 23 prospective studies that included 47,937 positive persons and bracketed the introduction of HAART showed the incidence of Kaposi's sarcoma to drop from 15.2% to 4.9% and lymphomas in general from 6.2% to 3.6% [9]. The major impact was evident in cerebral and immunoblastic subtypes, no difference in Hodgkin lymphoma with the contrasting relative increase in the Burkitt category [9]. From Europe a report of 7300 patients documented a dramatic decline in all illnesses defining the acquired immunodeficiency syndrome in response to antiretroviral therapy but, paradoxically, a relative increase in lymphoma [10]. These inconsistencies suggest a modest impact on overall incidence in survival that is not uniform across the different histologies and may reflect the concurrence of varying aetiologic agents or oncogenic processes. Variables such as viral load, low CD4+ T-cell count, poor performance status or increased age are established as associated risk factors leading to poor virologic control and correlating with significantly higher incidence of tumour development and inferior outcome following therapy [10]. Some of the latter may respond more favourably to immune reconstitution thus arguing for earlier commencement of treatment prior to development of severe immunologic decline [11]. On the subcontinent of Africa, the best statistics show that less than 25% of persons living with AIDS are receiving HAART [1]. Most of those on HAART have had an AIDS defining illness that results in the commencement of the antiretrovirals, which also implies that patients are presenting late when profound immunosuppression has been established.

Based on these facts it is anticipated that the expanded care and treatment program is not going to have a significant impact on the demographics of ADL for the time being in Africa. In South Africa there are estimated to be 5.5 million people living with HIV and the national prevalence rate suggests that 1 in 4 of the sexually active age group are infected with HIV [12]. A study conducted in one of the high prevalence provinces of South Africa, showed the incidence of ADL has increased four fold in the last 10 years, and this excess is attributable to HIV infection [13].

3. Pathogenesis and clinical presentation

Clinical heterogeneity likely reflects differing pathogenetic mechanisms including chronic antigen stimulation, release of cytokines such as IL-6, IL-10, CD23, CD30 and CD44 or co-existence of viruses such as HHV-8 and EBV with the latter virtually always demonstrable in primary central nervous system, diffuse large B-cell and Burkitt lymphoma and both together in plasmablastic (PBL) and primary effusion (PEL) [14]. Three major aggressive forms exist. Firstly Burkitt or Burkitt-like tumours are typically widespread with central nervous system involvement, expand rapidly and are associated with fever, night sweats and unexplained weight loss. Also of B-lineage are the PBL category while T-cell lymphomas remain rare while in PEL variants there is often a paucity of membrane markers. Other pathologic subtypes include polymorphic disorders resembling posttransplant associated lymphoproliferative disorders. Each of these differ in their underlying genesis, presentation and response to therapy but as a generalisation older age, low CD4+ counts
and no prior antiviral therapy favour development of these malignancies.

Available evidence supports a modest benefit for drug treatment on overall incidence in survival but this is not uniform across the histological spectrum suggesting that the presence of other aetiologic agents or oncogenic processes operating concurrently may respond variably to immune reconstitution. This concept would be consistent with the observation that these lymphoreticular malignancies have a different relationship here with the infection and its treatment to that seen with the defining entities such as Kaposi sarcoma.

Micro ribonucleic acids (miRNA) are a recently described class of non-coding molecules present in humans and eukaryotic organisms capable of modifying intracellular signalling by regulating message through antisense complimentary binding. Several hundred of these have been identified as being involved, directly or indirectly, with transduction or inhibition of cells cycling or apoptotic pathways [15,16]. Their presence during latent infection may lead to involvement with candidate proteins and here similarities as with EBV infections. Clarification of their role in these pathways may shed new light on the molecular pathology of cancer.

The human immunodeficiency virus may have direct carcinogenic activity through in insertional mutagenesis [17]. Retroviruses infect cells by direct host genomic contamination of viral cDNA which is a product of the reverse transcription of foreign RNA. This process can arise randomly but recently found to occur within the transcriptional unit as opposed to downstream or upstream in any particular gene leading to activation all deactivation of genes that modulate cell cycle or cause apoptosis and thus candidates for promoting malignant transformation [18,19].

4. Treatment and the modifying effect of HAART

Prior to the availability of effective antiretroviral drugs tolerance was poor and infectious complications high with complete response rates between 50% and 60% and two years survival being only 10% irrespective of the regimen employed [20,21].

Clinical trials have shown that a CD4+ count <100 [22] and 50/mm<sup>3</sup> [23,24] to be predictive of lower complete response (CR) rates. CD4+ lymphopenia has been associated with increased risk of infection in the neutropenic HIV infected patient [23].

The nucleoside reverse transcriptase inhibitor Zidovudine (AZT), besides its antineoplastic activity, has shown myelosuppressive effects and has been studied in ADI patients in combination with methotrexate [25]. It is generally recommended that AZT should be avoided during chemotherapy. In general, Protease inhibitor (PI)-containing HAART is well tolerated when administered e.g. with standard CHOP-like regimens, and their concurrent use can be recommended despite the fact that certain PIs such as indinavir may interfere with the metabolism of cytotoxic drugs, leading to increased intracellular levels [26]. Stebbing et al. [27] analyzed the impact of various types of HAART on the development of ARL and found that PI-based HAART and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based HAART were equally effective at preventing ARL. Whether coadministration of PIs with infusional regimens like cyclophosphamide, doxorubicin, and etoposide (CDE) or etoposide, doxorubicin, vincristine, cyclophosphamide, and prednisone (EPOCH) is associated with increased toxicity will have to be determined in ongoing and future trials. With the introduction of highly active antiretroviral combinations outcome has improved largely as a direct consequence of enhanced immunologic status and bone marrow function. One of the arguments speaking for the concomitant use is the fact that in the era of HAART, patients with profound immunodeficiency are clearly living longer, although not always with a reconstituted immune system [28,29]. Reasons for HAART omission include concerns of drug interactions with chemotherapy and inconsistent compliance with antiretrovirals resulting in increased resistance [30] which fuels the discussion whether antiretrovirals should be optimally administered concomitantly or immediately after completing the lymphoma therapy.

5. Subtypes

5.1. Diffuse large B-cell (DLBL) and Burkitt lymphoma (BL)

These two represent the majority of all AIDS-related lymphomas [6].

Ratner et al. [25] demonstrated the feasibility of combining CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy with concomitant HAART and reported CR to be 48% (full-dose CHOP) in comparison to 30% (reduced-dose
CHOP). Only one opportunistic infection was reported during chemotherapy administration.

Several groups have investigated the use of infusional chemotherapy regimens. When used in this way dose-adjusted regimen such as EPOCH [22] has been designed with the intention to correlate dose intensity with haematologic toxicity. Etoposide, doxorubicin and vincristine was given continuously as a 96-h infusion together with oral prednisone for 5 days. This was followed by an adapted dose of cyclophosphamide according to initial CD4 cell counts. CR was found to be 74% and overall survival (OS) after 53 months follow-up was 60%. Patients with an initial CD4 count >100 cells/mm³ had a better survival compared to patients with a CD4 count of <100 cells/mm³. Unfortunately the study was not designed for the concomitant use of HAART. Other infusional regimen such as CDE consisting of cyclophosphamide, doxorubicin, andetoposide emerged feasibly when given with HAART [31]. Infusional therapy was postulated to reduce the risk of multi-drug resistance by overcoming the drug efflux associated with multi-drug resistance-1 gene expression [32].

In Burkitt lymphoma remission and survival rates remain poor when treated with CHOP-like regimens whereas HyperCVAD, alternating with high-dose methotrexate and Ara-C, led to 92% CR in 13 individuals with BL with 31 months median duration of CR and survival of 12 months, respectively. Outcomes appeared to be better for those receiving HAART [33]. Interestingly no difference was found in HIV-positive and -negative patients receiving the PETHEMA LAL3/97 protocol (Prednisolone, Vincristine, Daunorubicin, Asparaginase, Cyclophosphamide and intrathecal therapy over 5 weeks). Complete remission in HIV-positive and -negative patients was achieved in 71% and 77%, respectively. The estimated 2-year OS was 51% and the only adverse prognostic factor was age of more than 60 [34].

As a general treatment recommendation for aggressive ARL infusional chemotherapy regimens are well tolerated and should be given preference to conventionally administered chemotherapy. Furthermore BL response rates and survival seem to be better when using intensive chemotherapy regimens and they are well tolerated especially when retroviral therapy is given concurrently.

5.2. Plasmablastic lymphoma

Plasmablastic lymphoma (PBL) is a subtype of large B-cell lymphoma more common in HIV-infected individuals often presenting extranodally in the oral cavity, jaw or rectum area [35] and has a rapidly progressive course. Characteristically large plasmablasts are found and there is close association with EBV [36] as well as HHV-8 [37]. In a study performed in the pre HAART era all patients followed up died within 34 months (median 7 months) [38]. This was contrasted by a post HAART era study [39] in which 8/12 patients were reported to be alive (median follow-up > 11 months) after treatment with cyclophosphamide, doxorubicin, high-dose methotrexate/ifosfamide, etoposide and high-dose cytarabine (CODOX-M/IVAC) – a treatment protocol originally designed for BL.

5.3. Primary effusion lymphoma (PEL)

This is a high grade, mature B-cell neoplasm that presents as a malignant serous effusion without solid tumour formation accounting for approximately 5% of all ADL and has been found to be associated with HHV-8 and EBV infection [5]. Outlook is poor despite the use of HAART with or without chemotherapy, attributable to presentation in very advanced stages of HIV disease and appears to be resistant to chemotherapy. Boulanger et al. [40] published results on 28 HIV-positive patients diagnosed between 1993 and 2003, (bracketing the time when HAART was introduced), with a CR rate of 50% and one year OS of 40%. It was concluded that performance status and use of HAART were the two most predictive prognostic factors.

In another small study 4 out of 5 patients were treated with chemotherapy and HAART while 1 out of 5 was treated with HAART alone achieving a CR in 3 patients [41]. Immune reconstitution is probably an important factor in the treatment of this disease.

5.4. Primary central nervous system lymphoma

This presentation is common in patients with severe immunosuppression defined as CD4 count less than 50/mm³ and a positive EBV status. Historically, prognosis is poor, with median survival rarely beyond several months duration but patients responding to HAART have demonstrated a prolonged median survival up to 1.5 years [42]. Among those receiving radiotherapy, completion of the prescribed antiretroviral course and treatment to at least 30 gray were both associated with better outcomes [43] but at the price of late complications
arising from irradiation such as leukoencephalopathy and radiation necrosis. Smaller reports in this subpopulation have also shown good responses to high-dose methotrexate-based regimens without the associated radiation related leukoencephalopathy [44]. The current available data favours treatment with high-dose methotrexate; radiotherapy should be reserved for relapse or chemoresistant disease. In both settings the addition of HAART is recommended.

6. The role of rituximab

This anti-CD20 monoclonal antibody has shown to improve response rates and OS in HIV-negative aggressive cases treated with CHOP without an increased risk of infectious complications [45]. In a phase III trial CHOP was compared to the same regimen and rituximab (R-CHOP) [24]. Rituximab at a dose of 375 mg/m² [2] was given with each cycle for a total of 6–8 cycles followed by maintenance in three monthly intervals. All patients received combination antiretroviral therapy and granulocyte-colony stimulating factor (G-CSF) support. Nonsignificant differences, marginally favouring the antibody containing arm were shown with CR rates of 58% versus 47%, progression-free survival 45 versus 28 weeks and OS 139 versus 110 weeks but with an increased risk in death from infection (14% versus 2%). In contrast, results from a trial using rituximab with infusional etoposide, vincristine, doxorubicin, and cyclophosphamide and prednisone (R-EPOCH) [46] have demonstrated a particular benefit for rituximab in patients with a CD4 cell count <100/mm³. Compared to historical controls, this agent improved survival from 16% to 57% at 19 months median follow-up. No additional benefit was seen for those with CD4 cell counts >100/mm³ but these patients already had cure rates of approximately 90%. Neutropenia was more frequently observed with R-EPOCH during and after chemotherapy but without any increased risk of death from infection.

More studies are needed until it can be suggested that rituximab will contribute to improved control in ADL but obviously also at a higher risk of severe infections and should be therefore used with greatest caution or only in controlled studies in patients with severe immunosuppression. It is possible that infectious complications may be reduced with routine fluoroquinolone prophylaxis during periods of severe neutropenia. The role of rituximab requires further clarification prior to routine use in patients with ADL.

7. High-dose chemotherapy and transplantation

Patients with relapsed or refractory ADL should be considered for high-dose chemotherapy followed by autologous stem cell transplantation (ASCT) [47] if appropriately selected. Even though all patients were started on HAART only the minority of the patients were able to continue antiretroviral treatment. With 32 months median follow-up OS was 85% and progression free survival 81%.

Infectious complications during ASCT were similar to those noted in the HIV-negative population but larger studies with longer follow-up are needed to better define the role of ASCT for the treatment of persistent or relapsed lymphoma. Krishnan et al. [48] reported that between 1998 and 2006, 28 HIV positive patients with high-risk ADL underwent ASCT with a median follow-up of 41 months, 2 year OS of 78% and PFS of 78%. All engrafted with median time of 11 days to absolute neutrophil count (ANC) > 0.5 × 10⁹/µL. Median HIV viral load (VL) at ASCT was 6113 copies/ml with 22 having an undetectable VL. At two years only 9 patients had an undetectable VL. Median CD4 count at ASCT was 164 cells/mm³, rising to 263 cells/mm³ at two years post ASCT. Despite the excellent outcome and ongoing antiretroviral therapy this demonstrates clearly, there is still progressive attrition of the immune system and reflects the natural history of HIV infection and limitations of current antiviral therapy.

8. Socio-economic impact of treating arl patients in africa

The approach in treating ADL patients is also dependent on the financial constraints under which a health system has to operate. It is therefore not feasible to drain the budget in a low resource setting just because certain treatment modalities exemplified by monoclonal antibodies such as rituximab or ASCT have been found to be superior over other but maybe more affordable therapies. As a consequence arising from these financial limitations patients with ADL would have to be firstly stratified with reference to their potential risk for developing opportunistic infections caused by pneumocystis carinii, cytomegalovirus, atypical mycobacterium species or others. Several authors have confirmed
that ADL patients were less commonly diagnosed with opportunistic infections in the HAART era as compared to pre HAART era patients [49,50].

Therefore we would suggest to design future studies or risk stratify treatment decisions in ADL patients with regards to their CD4 cell count. This would mean to treat patients with a higher risk for opportunistic infections defined by CD4 cell counts <100 cells/mm³ with infusional regimens such as dose-adjusted EPOCH or CDE under the concomitant protection of HAART. Patients with a low risk for developing opportunistic infections here with CD4 counts >100 cells/mm³ where toxicity or treatment-induced infections are less problematic could receive full or even high-dose chemotherapy determined by the subtype.

In addition, the threshold to prescribe prophylactic fluoroquinolones during periods of neutropenia should be low and ADL patients undergoing chemotherapy should receive prophylaxis for Pneumocystis carinii pneumonia regardless of their CD4 cell count.

9. Summary

Despite the historically poor prognosis in ADL, outcomes have been improving with the use of antiretroviral therapy and more intensive chemotherapy regimens, especially in aggressive subtypes. Particularly in patients with CD4 cell counts <100/mm³ relapse rates remain high and overall prognosis poor. Infusional chemotherapy regimens such as EPOCH or CDE have been designed especially for patients with aggressive subtypes and should be given preference especially in patients with a CD4 cell count <100/mm³. These regimens are generally well tolerated achieving complete response rates in aggressive lymphoma patients in 50–75% and 2–3 years overall survival in 40–60%. The use of either concomitant, or sequential administration of HAART, depending on absolute neutrophil and CD4 cell counts remains to be clarified in future studies even though there is a preference for concomitant HAART in patients with a CD4 count <100/mm³. Burkitt lymphoma should be treated with intensive regimens rather than standard CHOP-like chemotherapy. Independent from CD4 count the neutropenic patient should additionally receive prophylaxis against the most common bacterial and opportunistic infections.

The addition of rituximab to chemotherapy may have a role which needs to be further clarified in future studies although it seems that infectious complications appear to be higher, notably for those with severe immunosuppression. Once with refractory or relapsed disease, high-dose chemotherapy with autologous stem cell transplantation, where affordable, appears to be well tolerated and effective but should be considered for selected patients and remains an investigational approach.

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References


Biology of the Immune Paralysis and Haemato-vascular Complications seen in Human Immunodeficiency Virus Infection

by

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Abstract
Retroviral infections have reached pandemic proportions in sub-Saharan Africa and are cutting across continents with rapid and devastating effects. The initial focus on immunologic defects and primarily infectious complications continue to be modified by highly active antiretroviral therapy. A greater understanding of the pathobiology of this formidable foe has opened many opportunities for interventions as the battle continues to contain and hopefully find a long lasting compromise between infectivity and mortality of this group of viruses and their human hosts. As acute becomes a more chronic disorder longer survival allows specific changes on target organs including blood and bone marrow function and the vascular tree. This relationship is given consideration in this review.

Key words:- HIV-1 geneology, CD-4 depletion, T-cells, cardiovascular complications.

Introduction
Since its discovery in 19831,2,3, Human Immunodeficiency Virus (HIV) has been responsible for approximately 25 million deaths worldwide. There are estimated to be 47 million people living with HIV worldwide, 25 million of which live in Sub-Saharan Africa4. A further 45 million are projected to become infected before the end of 2010 unless a drastic global prevention program is instituted5. Classified as a pandemic, current mathematical models are projecting an escalation that is likely to peak around the middle of this century with a shift in its current epicentre from Southern and West Africa to new foci in Asia and Russia. A staggering anticipated burden of disease in the range of 60 to 240 million infected people will be living in India and China by 20506. This represents
a devastating psychological impact on the regions that are and will be most affected by this phenomenon with escalating human suffering and profound economic retardation. Unspiring and transgressing all specialities of Medicine, it is incumbent on us to behave this unprecedented scourge with the compassion and professionalism it deserves. Never in the history of mankind has there been such an unrelenting multifaceted impact on human welfare, which threatens to transcend into posterity in all its ramifications. This review attempts to put in perspective the lessons learnt from the outset of this disease, and demonstrates succinctly how it transcends multidisciplinary fields by highlighting the understanding of the impact is has on the haematopoietic and vascular system. Without an effective prophylactic vaccine or some form of immunogenetic modulation, it is likely to exponentially progress and change dynamics around the world.

**HIV-1 Geneology**

HIV is a retrovirus belonging to the lentivirus group, capable of infecting susceptible human cells and incorporating reverse transcriptase derived complimentary DNA into the host cell genetic banks, thus starting of a process of self-replication using host machinery resulting in progressive immunological decay and ultimately, Acquired Immune Deficiency Syndrome or AIDS. There are 2 known main groups HIV-1 and HIV-2. The largest group HIV-1, is further subdivided into subgroups M, N and O. HIV-1 subgroup M is responsible for the demographic holocaust and global pandemic that humanity is facing currently. There are now numerous clades and hybrids of group M that have found unique niches in human populations around the world due to a complex interplay between viral characteristics, human genotypes and social demographics. HIV is closely related to other lentiviruses known as the Simian Immune Deficiency Viruses (SIV) found in African primates of which there are almost as many subtypes as there are species in Africa, thus demonstrating a high degree of evolutionary specificity. The Sooty Mangebey African monkey and Chimpanzees harbour alternative immunodeficiency viruses known as SIVsm for Sooty Mangabey and SIVcpz for Chimpanzee. These are the closest relatives of the HIV group. Within the HIV group of viruses, the HIV-1 group N and the HIV-2 groups C–G are the closest genetically to SIVcpz and SIVsm respectively (Figure 1).

![Phylogenetic tree of the SIV/HIV lineages](image)

Figure 1: Phylogenetic tree of the SIV/HIV lineages. SIVpz refers to the SIV virus found in chimpanzees and SIVsm to the SIV found in a specific African Green Monkey known as Sotty Mangabey. The phylogenetic tree shows the probable relationship between the SIV and HIV in terms of evolutionary similarity that these viruses have to one another and points of possible divergent ancestry.

Both of these groups are extremely rare in humans. Only six persons are known to have been infected with HIV-1 group N and only single individuals by HIV-2 groups C–G. This may however
represent the beginning of the historical evolutionary process of cross-species adaptation of SIV to HIV (7). SIV infection in African monkeys appears to be ancient and a well-adapted lineage specific process resulting in a state of non-pathogenic latency in most cases. This is not true for humans infected with HIV or Asian macaques infected with SIV. Both of these hosts are highly susceptible to these viruses and develop a pathogenic state slowly culminating in AIDS.

The First Established Cases of HIV-1 and the Timing and Location of its Origins

The first well recognized epidemic of HIV was in the early 80's among gay men living in San Francisco. However with the aid of retrospective insight, several conclusive public health scenarios confirmed that pockets of small epidemic cases of HIV infection and AIDS existed much earlier in Africa and Europe. Conclusive support for this is found in numerous reported cases such the Norwegian sailor who had travelled extensively in Europe and Africa in the late 1950's and early 60's, and subsequently developed the characteristics that are now known as HIV/AIDS in 1966, his wife was also affected as was their child born during this period. All of them died around 1976. Frozen serum from all three of them retrospectively tested positive for HIV-1. This suggests that the virus must have been in circulation and contracted by this sailor well before 1966. There are numerous similar accounts of patients and sera examined from people living or having contact with Central and East Africa before the outbreak of the American epidemic. Work done by Dr Ho and his team at the Aaron Diamond HIV centre in New York, identified conclusively the presence of HIV-1 in a sample of stored frozen serum from a man that lived in the Democratic Republic of Congo the former Belgian Congo in 1959. Thus making this the first ever-confirmed case of HIV-1 infection in Man. These facts demonstrate that HIV as a human infection has been around for a much longer time frame than we are accustomed to thinking about and Africa probably has the most mature epidemic to date, which would explain why the statistics are so alarming in this geographical location.

Immuno-Biology of HIV Infection

The Trojan Horse Phenomenon:

HIV infects susceptible human cells by mechanism that involves a combination of several receptors, co-receptors and surface antigens. Most importantly among these receptors are the CD4 and the chemokine receptors CCR5 and CXCR4 on a variety of host cell, and the glycoproteins gp120/gp41 complex on the HIV surface. The first cells to encounter the invading virus are usually the tissue macrophages or dendritic group of cells (DC). Majority of naturally occurring strains of HIV-1 use CCR5 as a co receptor for primary infection of both the tissue DC and macrophage cells. The HIV virus has been observed to preferentially target and infect fixed tissue or circulating DC precursors using the CCR5 receptor.

Infection of DC by the virus generates a cytokine induced intrinsic up-regulation of viral expression in and on the cell thereby increasing the transmission potential to other susceptible cells in the microenvironment by migration and chemo-attraction. By another mechanism, the DC will readily attach and bind to the virus gp120 envelope glycoprotein with several adhesion molecules. Among these is DC-SIGN (or CD209), a C-type (Ca-dependent) lectin that selectively recognizes high-mannose oligosaccharides of the virus gp120. DC-SIGN is expressed on some DC subsets, including those derived from blood monocytes and particularly those found in lymphoid tissues and beneath genital mucosal surfaces. DC-SIGN-expressing cells can internalise HIV virions via the CCR5 and CD4 receptor complex into a trypsin-resistant compartment, sequestered from the immune system and where viral infectivity can be retained and amplified for several days with the effect of potential enhanced onward transmission as it migrates through tissue boundaries. The virus is therefore transported to the central lymphatic tissues by the DC either internalised in an intact and infective form, on the surface or digested within the context of the MHC molecules for cell mediated response.

Conjugates between DC and T helper cells are easily formed, a process which is designed to provide cross talk between innate and adaptive compartment of the immune system in the antigen presenting process. HIV with the help of the DC uses this functional liaison with extreme prejudice serving to facilitate transmission by locally concentrating whole virions in the proximity of its main target cells during this physiological interaction of the infectious synapse. The DC thus acts to
bring the intact and infective enemy into direct contact with the most strategic cells of the immune system. But as fate would have it, these cells are the precise victims of the virus. The DC can thus be described as the Trojan horse of the invading enemy and inadvertently facilitating the rapid dissemination of a highly destructive virus, which has a crippling effect on the immune system\textsuperscript{18}. HIV infected DC, cell macrophages and monocytes by virtue of their mobility and migratory capacity will act as the transferring medium of the virus to sites remote from the point of initial infection. By this same modality transmission also occurs into highly susceptible lymphoid rich tissue spaces and other organ spaces such as the bone marrow and sanctuaries like the brain\textsuperscript{15,16,17,18}.

**CD4 Depletion and Factors Fuelling the Flames of Immunological Attrition:**

T-lymphocytes are divided into two broad categories which are the newly thymic released naïve cells and antigen experienced also known as memory cells. Within this compartment two further subtypes are recognised. These are the central memory (CM) T cells characterised by a CCR7 positive and CCR5 negative surface phenotype which circulate between the peripheral blood and the lymph nodes (LN) and the effector memory (EM) T cells that migrate out into the extralymphatic sites like the gastro intestinal (GI), genital lamina propria and the skin.

![Diagram](image)

**Figure 2:** T helper cell genealogy and homeostasis. Thymic derived cells replenish the naïve compartment, which in turn is cytokine driven to retore the central memory compartment confined to the lymph nodes and blood. This compartment is somewhat resistant to HIV infection by virtue of their chemokine receptor profile and important in replenishing the effector memory compartment, which resides predominantly in the mucosal surfaces of the gut and respiratory systems. This compartment is highly susceptible to HIV infection and undergoes dramatic depletion in the early and chronic stages of HIV infection.

These subsets are CCR7 negative and CCR5 positive which confines them to the periphery where they will remain and die. The EM cells are believed to constitute about 50% of the T population and are exquisitely vulnerable targets of HIV by virtue of their CCR5 coreceptor surface phenotype. Infection of these cells via the CCR5 receptor is designated R5 viral tropism\textsuperscript{19}. The EM pools are rapidly and almost completely depleted in the acute infective phase and attempts to replenished them from the CM compartment occurs which in turn is dependent on the thymic naïve population output\textsuperscript{19} (Figure 2).
There is convincing evidence now that this process of replenishing of EM cells from the CM compartment is cytokine driven and the rate at which this occurs has clinical implication for the progression state and natural history of the disease. Evidence suggests that rapid progressors have a deficient capacity to replenish the EM T cell compartment because of a physiological cytokine deficiency of IL-2. Resting CM and naïve T cells do not express CCR5 and are thus spared the devastation in the acute phase infection. If one then just defines the effect of the virus on T lymphocytes by examining the peripheral blood (PB) alone, a false impression emerges of the state of T helper destruction going on in the peripheral sites. However later in chronic phase when there is an exhaustion of CCR5 susceptible cells and replenishment is less efficient, HIV is able to mutate into an R4 tropic virus, which has an increased propensity to utilise the CXCR4 chemokine coreceptor as the main means of transfecting cells. CXCR4 is found most readily in resting CM and naïve cells resulting in depletion of the CM pool. R4 tropic viruses therefore become the predominant quasi-specie towards the end of the infection consuming what is left of the host T cell repertoire leading rapidly to AIDS. All T memory cells can undergo activation with upregulation of the chemokine receptor CCR5 at any time in their life span and can then be identified by characteristic additional immunophenotypically markers CD45RO and high CD26 expression. In this activated state the cell become even more susceptible to HIV infection. The upregulated mRNA expression of several chemokine receptors on activated memory T cells has been described as a phenomenon that appears to be dependent on exogenous cytokines. This heightened state of persistent activation can occur as a direct consequence of the HIV infection itself or independent of HIV by virtue of the environment or life style of the individual. Such a scenario would be found in tropical environments where parasitic, bacterial and viral infections are endemic and populations have become long term chronic carriers of these infection, or suffer repeated multiple infections over time, exacerbated in locations where instability is precipitated by periods of seasonal malnutrition or political upheaval. A similar situation would be found in intravenous drug abusers and men who have sex with men. Activated EM T cells represent the lymphocyte subset showing a higher transendothelial chemotactic potential, rapidly migrating to a site of inflammation and with the highest susceptibility to HIV infection. This setting only results in a ready and sizeable pool of activated competent CD4 EM cells meeting a formidable fee, which will lead directly and indirectly in their demise. Attempts to minimize the destructive effect of what appears to be a rapidly cascading immune and cytokine storm occurs through the amplification of regulatory pathways such as the CD4+ CD25+ T regulatory cells particularly at the mucosal surface where the destruction of the EM cells is so pronounced. This could have a beneficial effect by dampening down the activation state, which is the environment in which the virus flourishes. Now that it is apparent how much of the early and chronic phase damage to the T helper population takes place in the peripheral tissues of the mucosal surfaces of structures like the GI and lungs it is easier to appreciate why examination of the PB and the LN does not reveal much attrition at these stages.

The majority of GI tract CD4 EM cells expresses CCR5 and comprises a higher frequency of activated T cells than in the PB or LN in HIV-uninfected and infected individuals, thus representing the ideal targets for sustained HIV replication. Direct infection of this population results in an irreversible and profound depletion during the acute and chronic phase of HIV disease. However what is not yet fully understood are the direct or indirect mechanisms that the virus uses to drive a state of persistent activation of the immune system. This defines the chronic phase of the infection, a phenomenon resulting in far reaching consequences culminating in sustained attrition and aberrations of lymphoid tissue architecture that in turn impacts upon the ability of lymphoid tissue to support normal lymphocyte homeostasis and antigen presentation.

A similar phenomenon is seen in the primate macaques pathogenic model where primary simian immunodeficiency virus (SIV) infections result also in the dramatic depletion of both CD4 CCR5 EM T cells as well as a state of chronic unquenched cytokine storm. Eventually, persistent SIV replication results in chronic-phase immunological collapse or macaque AIDS. Again the main catalyst for this phenomenon appears to be persistent activation of the immune system. Data suggest that although CD4 EM T cell depletion is largely determined by viral and immunological mechanisms, it is exacerbated by failing production from a chronically progressive decline of CD4
CM T cell pool\(^2\). In the acute infection, GI CD4 EM T cells harbour on average, 13-fold higher HIV-1 viral DNA levels and 10-fold higher HIV-1 RNA levels than PB CD4 T cells. HIV-1 RNA is detectable in both "activated" and "non-activated" mucosal CD4 T cells of the GI although a significantly higher number of infected activated and proliferating T cells were detected in the GI tract compared to the PB.

A robust HIV specific cytotoxic response is detected in the GI tract as early as 18 days into the acute infection. Mucosal pan CD4 T-cell depletion is therefore probably multi factorial resulting from direct acute and chronic viral assault of susceptible activated CD4 T cells, which is compounded by activation-induced apoptotic cellular death and an initial robust host cytotoxic cellular response targeted against any infected cells\(^3\).

Interestingly this pattern and kinetics of early, severe and persistent dramatic loss of CD4 EM T cells in the GI tract has been found to occur in both pathogenic and non-pathogenic lentivirus infections. The African monkeys being the natural host, exhibit a chronic latent infection with insignificant consequences, while humans and the Asian macaques develop progressive immunological decay and AIDS. Although the non pathogenic SIV infections in African monkeys induces an almost similar pattern of mucosal target cell depletion observed during pathogenic HIV infections, this depletion seems to occur in the context of limited local and systemic immune activation.

The extent of local and systemic immune activation is the distinguishing feature between the pathogenic and non-pathogenic lentivirus infections\(^4\). It is suggested that in the disease-resistant SIV-infections of natural host monkeys of Africa, evolutionary adaptation has occurred to preserve immune function with a less vigorous ablation of mucosal CD4 EM T cells and an attenuation of the immune activation follows the acute SIV infection protecting these animals from progressing to AIDS\(^5\). Reports reveal that the SIV natural hosts and their specie specific viruses have co-adapted to exist in a non-deleterious state by expressing lower levels of CCR5 on their CD4 T cells isolated from blood, lymph nodes, and mucosal tissues, and perhaps higher regulatory suppression\(^6\). It would appear that perhaps as a consequence of this, the mucosal depletion observed in the natural host may be less functionally devastating when compared to that seen in human HIV infection and the SIV AIDS model of the macaque.

**Consequences of Mucosal Specific Depletion of CD4 Effector Memory T-cell Population:**

It is proposed that the large-scale and rapid depletion of CD4 EM T cells in the weeks after acute HIV infection which occurs predominantly in the GI tract as described above, leads to an impaired intestinal integrity allowing the systemic translocation of gut microbes, or some of their constituent components. Circulating levels of these constituent components are identified as bacterial lipopolysaccharide (LPS), which can be used as a marker for microbial translocation and are found to be markedly elevated in the sera of chronically infected HIV individuals and in macaques experimentally infected with SIV. LPS is known to activate both innate immune cells such as macrophages and adaptive immune T and B cells via Toll Like Receptor (TLR) signalling pathway on the surface of these immune cells\(^7\). This increase in circulating LPS corresponds directly with footprints of immune activation. In contrast nonpathogenic SIV infection of natural SIV infection in African monkeys, microbial translocation does not seem to occur despite the fact that GI tract appears to have undergone similar depletion of CD4 EM T cells. Nonetheless, these findings, although conflicting in our current understanding, does however suggest a possible mechanism for chronic immune activation in the context of a compromised gastrointestinal mucosal surface integrity\(^8\). Further more TLR have also been shown to be activated by free single stranded HIV RNA ligands, which induce T-cell activation. HIV-1-encoded TLR ligands may, therefore, directly contribute to the immune activation observed during viraemic HIV infection\(^9\).

In chronic HIV infection, most untreated patients will eventually also start to lose naïve CD4 T cells as the virus becomes more R4 tropic\(^10\), whereas a minority of patients will preserve the naïve pool despite persistent high viraemia. Although antiretroviral therapy (ART) mediated viral suppression generally results in a rise of naïve and total CD4 T cells, certain patients experience very little or no T-cell reconstitution. Data shows that the inability to sustain a naïve pool from which the memory cells are replenished is partly the result of a sustained thymic defect and is clearly shown in some patients, whereas efficient thymopoiesis will support both naïve and memory cell
compartments with a slower progression time to AIDS. In the majority of ART-treated patients, CD4 T-cell recovery is associated with the normalization of thymopoiesis, demonstrating that efficient thymopoiesis is a key factor in the natural maintenance and recovery of naive and total CD4 T cell compartments.\textsuperscript{34}

**Human Genetic Polymorphisms and HIV Vulnerability:**

CCR5 is the obligate co-receptor for infection by SIV and most transmitted forms of HIV. Humans who lack CCR5 expression (CCR5Δ32) are highly resistant to HIV infection.\textsuperscript{22} Cohort studies indicate that individuals that are homozygous for the CCR5 mutation are almost completely resistant to infection by CCR5-tropic strains of HIV-1. To date, there have been only eight reports of HIV-1 infection in CCR5Δ32 homozygous individuals. These individuals appeared to have been infected with a CXCR4-tropic strain of HIV-1 and did develop AIDS. Individuals that are heterozygous for CCR5Δ32 are infected at rates similar to CCR5+/CCR5+ individuals. However, their disease course is prolonged, presumably because of reduced efficiency of propagation from DC's to susceptible cells via the infectious synapse.\textsuperscript{35}

It is possible that a selection for low expression of CCR5 in humans in high HIV prevalence areas could evolve as one mechanism to escape the pathogenic consequences of infection. In contrast to lentiviral infections of humans and macaques, SIV infection of natural hosts is non-pathogenic despite high levels of viral replication. However, the mechanisms underlying this absence of disease are unknown. Reports reveal that natural hosts for SIV infection express lower levels of CCR5 on CD4+ T cells isolated from blood, lymph nodes, and mucosal tissues. Given that this pattern of low CCR5 expression is found in 5 different species of natural SIV hosts (sooty Mangabeys, African green monkeys, mandrills, sun-tailed monkeys, and chimpanzees) but the converse in 5 non-natural/recent hosts (humans, rhesus, pigtail, cynomolgus macaques, and baboons), suggests it may represent a key feature of the co-evolution between the virus and its natural hosts that led to a non-pathogenic infection. Beneficial effects of low CCR5 expression on CD4+ T cells may include the reduction of target cells for viral replication and a decreased homing of activated CD4+ T cells to inflamed tissue.\textsuperscript{30}

Should HIV pandemic infection persist uncontrolled in human populations, we might observe a bottleneck in the human race positively selecting for genotypes that confer survival advantage to the host in a similar way to what has occurred in African primates. The Chemokine receptor CCR5 is apparently dispensable for normal human growth, differentiation, and immune functions, despite what appears to be a major function in the rallying up of immune competent cells to sites of infection. Among the Caucasian populations, the occurrence of a CCR5-null allele, known as CCR5Δ32, is \(\approx 10\%\).\textsuperscript{22} The CCR5Δ32 allele is a deletion resulting in a frame shift truncation of CCR5 that prevents the mutant protein from appearing on the cell surface. Individuals homozygous for the CCR5Δ32 do not show any apparent adverse phenotypic effects. In heterozygous CCR5Δ32 individuals, cell surface CCR5 is reduced to 20-30% of wild-type levels.

**Immune Disarray is Central to the HIV Pathodynamics:**

The progressive but multi-faceted disruption of both the humoral and cell mediated immune system is orchestrated via several mechanisms. The unrelenting decline of the T CD4 cells is as a result of a combined effect of direct infection, induction of apoptosis, syncytial formation, attrition and eventual thymic failure to replenish the naive pool from which the central memory and effector memory compartments are derived.\textsuperscript{35} The result is immunological memory loss that has been built up over the lifetime of the infected person through antigen encounter and T cell receptor rearrangement. HIV also keeps the humoral compartment in a state of disarray, by mechanism that can only be described as a cytokine storm. This is characterised by a state of activation of the B cell compartment with the characteristic polyclonal hypergammaglobulinaemia.\textsuperscript{16,17} There is evidence to suggest that a reduction of memory B-lymphocytes occurs in HIV infection which correlates with the defective humoral immunity observed and that hyperactivated naive B cells may represent the source of abnormal IgG production.\textsuperscript{38} Cytotoxic cell activity does not escape the brunt of HIV, and
CD8 cells suffer early senescence, which can be measured by telomere length and telomerase activity. Evidence suggests that chronic stimulation of T cells, such as that which occurs in HIV infection results in the development of CD8 T cells reaching a state where they are incapable of cell division much sooner.

Such a failure to proliferate is generally attributed to replicative senescence resulting from a state of continual stimulation as exists in HIV. This is also described as clonal exhaustion and may be one of the handicaps within the host that underlie the inability of cell-mediated immunity to suppress virus adequately.

**Biology as it Relates to Haematovascular Complications:**

The bone marrow microenvironment is profoundly disturbed by this cytokine storm, and is characterized by dysplastic haemopoiesis and multiple cytopenias so frequently seen in HIV infection and AIDS. Although the pluripotent stem cell which expresses both CD4 and CxCR4 co-receptors is resistant to HIV infection, the more committed lines along myeloid, lymphoid and megakaryopoietic cell lines, are highly susceptible to infection with HIV with resultant ineffective haematopoiesis. Clarity of the state of hyperactivation of the immune system as described above that underlies the chronic infection of HIV helps create a better understanding of the factors that promote atherosclerosis and vasculitic phenomena through elaboration of proinflammatory cytokines and interleukins. There is emerging evidence that the vascular tissues as well as corresponding coronary endothelium show significant nitric oxide damage in HIV infection due to excessive oxidative stress. This could provide important opportunities for interventional strategies aimed at reducing the risk, consequences and damage of this oxidative environment on the vasculature.

C-reactive protein levels are higher in HIV patients than in control subjects and there is a strong association and an elevated risk of cardiovascular events. Macrophages and monocytes are also in a state of heightened activity with a potential to migrate to the subendothelial space where they begin phagocytosis of lipoproteins to become foam cells, an early step in atherogenesis. Coagulation abnormalities as well as enhanced platelet activation that would predispose to thrombo-embolic events are well described in HIV patients.

Protein S deficiency is the most common, reported in 73% of HIV-infected men. Serum levels of von Willebrand factor are higher in untreated HIV patients than in control subjects, reflecting endothelial activation.

**Summary**

The pathodynamics of HIV infection in humans is as much a puzzling phenomenon now as it was decades ago at the outset of its discovery. The enigma of this cross-specie infection defines the limitations of our understanding of the whole picture of how man and nature interact and how parasites and host vie for survival of the fittest in a way that ensures genetic preservation of species into the future. At the moment HIV is certainly a young infection still trying to find a happy medium of balance between viral fitness and virulence. Certainly a peaceful cohabitation of virus and host is the ideal circumstance allowing the host to survive to procreate and the parasite the opportunity to be transmitted from one individual to another by adapting to the idiosyncrasies of communities. The current state of affairs is disadvantageous to both infective agent and host. In the African monkey, which is the natural host, mutual adaptation has taken place over many centuries in order to reach a symbiotic chronic non-pathogenic latent infective state. In the meantime, man is suffering tremendous attrition from this HIV plague and its ramifications are all encompassing. No discipline in medicine escapes the wrath of this slow virus on the human physiology, as it slowly chips away at the host’s ability to ward off other opportunists in the environment and simultaneously creating an immunological storm with multiple consequences. We describe in this review the latest concepts on the mechanisms of how HIV is able to efficiently and without fail destroy its host through immunological decay and cytokine induced chronic activation leading to eventual physiological exhaustion and systemic damage through oxidative pressure, with particular reference to the impact on the haematopoietic and vascular systems.
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Blood Transfusion
Thoughts on Component Therapy

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The transfusion of blood is, to a large extent, concerned with oxygen transport. Over the years an unfortunate tendency has developed in that doctors attempt to correct any deficit in this latter function by the non-selective practice of administering whole blood. Indeed, it has been said by a variety of people, many who should know better, that this approach remains the best way to remedy the problem. But is this really true?

Let us consider first the physicans. Smiley (1972) reported that 30 per cent. of patients in one Canadian centre who needed blood had this provided in the form of pack cells—the remaining two thirds were given whole blood. It is significant that, on the request form, red cell concentrate required special motivation and understandably busy doctors simply checked the box for whole blood and thus avoided extra paper work. In an attempt to determine whether the physicians ordering the blood really understood the problem, the survey was extended for a further period in which it was the whole blood that required motivation. Under these circumstances not one request for whole blood was received in the next year. These observations reflect an indifference on the part of physicians to the advantages that packed cells may have over whole blood rather than any firm conviction that the latter itself was superior for the purpose of increasing oxygen-carrying capacity.

Is it possible that a similar situation could exist in the surgical services? It is generally recognized that blood lost at operation may lead to a hypovolaemic shock and it has been said that the best way to correct this deficit is to restore the blood volume and simultaneously correct oxygen-carrying capacity with whole blood. In both clinical situations a similar response is obtained where the whole blood or red cells combined with crystalloids are administered in appropriate volumes.

If these observations are correct, then the continued use of whole blood in surgical departments may also stem from failure on the part of surgeons to recognize advantages that may result from component therapy.

It is interesting that when this matter is discussed among surgeons it is argued that the most physiologic way to replace the loss of blood is with whole blood. The very fact that ACD is being used, on the assumption that it is physiologic indicates a poor grasp of the situation. It is known that fresh whole blood has a pH of 7.0 and contains 3 to 4 mEq/l potassium while the same product, which has been stored for 21 days, has a pH of 6.5 and contains 16 to 17 mEq/l potassium. In this way the latter product resembles blood taken from a ketotic diabetic with renal failure. In small volumes dilution in the patient’s circulating blood minimizes these effects but large volumes may result in serious metabolic defects.

A second point frequently raised by surgeons in defence of whole blood is that the plasma provides substances necessary for haemostasis including platelets and coagulation factors. This argument is also tenuous since there is a disproportionate requirement of red cells and haemostatic material and it is clearly more logical and effective to provide the necessary erythrocytes as packed cells coupled with other components selected specifically to control the particular bleeding disorder. To illustrate this point we might consider the patient with haemophilia who is actively bleeding. It is known that reliable haemostasis cannot be achieved with the amounts of Factor VIII being infused in the volume of whole blood that will adequately correct the deficit in red cells.

Using these arguments a good case can be made out for component therapy since metabolic complications of acidosis and hyperkalaemia can be reduced while other valuable components in the plasma may be salvaged for more selective use (Beal, 1973).
In this regard it should be appreciated that the shelf-life of whole blood as well as packed cells is 21 days at which time 70 per cent. or more of the cells in the unit collected and stored in ACD will survive normally in the recipient. However, this approach does not take into account wastage that will occur when supply exceeds demand and, here again, component therapy can contribute to the more economical use of blood.

Methods are currently being investigated for freezing and storage of red cells which, when preserved in this way, may be reconstituted after months or even years of storage and immediately used without deterioration in their oxygen-carrying capacity. It is recognised that the cost of such a unit of packed red cells must be taken into account when their commercial use is suggested. Although the cost will initially be somewhat high cells are available to meet demand when supply is less freely available. When calculated to take this fluctuation into account, the cost of frozen cells may well offer benefits once local production was established. There are, in addition, other important reasons why blood stored in this way might benefit the community. In the first instance rare blood groups could be preserved for long periods of time and, secondly, there is now evidence of a decreased incidence of transfusion hepatitis in patients after exclusive use of this product (Carr et al., 1973).

In addition to simply raising the haematocrit much recent work has focused on the effects of storage on the capacity of the infused cells to deliver oxygen normally. Thus it has been demonstrated that storage in ACD decreases the intraerythrocytic content of inorganic phosphate and this parallels increasing affinity of haemoglobin for oxygen. Although it has been suggested that this metabolic defect rapidly corrects itself, the transfusion of large volumes of stored blood may well raise the haemoglobin to normal levels while oxygen delivery, the essential function of the red cells, may be inappropriately or even dangerously low. Appreciation of this point is of special significance in exchange transfusion in the newborn or when massive haemorrhage requires the infusion of large volumes of blood.

In addition to red cells, component therapy is being increasingly developed to provide concentrate of many other blood fractions (Medical Letter, 1972). Their proper use can no doubt refine the way in which the requirements of the individual patient are best met.

The treatment of various haemorrhagic disorders lend themselves ideally to component therapy and the treatment of haemophilia, for example, has undergone significant change in recent years. Originally fresh plasma was used but it was soon appreciated that the antihaemophilic globulin retained its function well when frozen in the fresh state. This preparation remains a popular form of therapy although cryoprecipitate is being increasingly used particularly where large volumes of plasma need to be administered. At the present time special concentrates of even higher activity are being developed while, at the same time, many of the other plasma fractions such as albumin, gammaglobulin, fibrinogen and hyperimmune sera are all emerging as components with real benefit in clinical practice (The Lancet, 1972).

Platelet dysfunction, whether qualitative or quantitative, may also give rise to a bleeding disorder. Thrombocytopenia occurs not infrequently following severe bone marrow damage after the ingestion of drugs including the antibiotic Chloramphenicol. Of special interest has been the problem created by aggressive treatment of a variety of malignancies, including acute leukaemia (Jacobs, 1973) where non-selective chemotherapy produces a varying degree of marrow damage. In these patients intensive haemostatic support with platelet concentrates is essential if optimal amounts of myelosuppressive drugs are to be given. It is important to appreciate that considerable expertise attends the preparation of the platelets and it is mandatory that rigid quality control be effected if the clinicians are to depend on the product supplied. This should include measurements of rise in platelet count after infusion as well as documentation of platelet survival in the individual patient. It is this latter type of requirement that necessitates close co-operation between the blood transfusion services and the clinician.

In addition to the various components described above, increasing interest and clinical dependence centres on the provision of granulocyte-rich suspension. In many situations transient bone marrow depression occurs and this manifests with agranulocytosis. Blood fractionation makes possible the provision of functionally active granulocytes ideally suited for use in these circumstances. In the severely immunosuppressed patient, such as the individual receiving cytotoxic therapy for acute leukaemia, defense mechanisms are severely
compromised and infection presents a particular problem. Much of the advance in the more aggressive treatment of these individuals has been made possible by the availability of this component. The introduction of blood fraction separators into the clinical service will greatly enhance the flexibility and the versatility with which the various blood components may be harvested and used. In centres where these are already available significant improvement in patient survival has been reported. Under normal circumstances, removal of granulocytes from a normal donor results in a small yield while the use of patients with chronic granulocytic leukaemia provides large numbers of functionally excellent cells but the latter approach remains, at this time, an experimental form of therapy.

Blood fraction separators have numerous other advantages particularly where single components of a rare blood type may be frequently required. Thus, in the patient with irreversible drug-induced bone marrow aplasia, who is being considered for bone marrow transplantation and in whom isoantibody formation should be avoided at all costs, the careful use of selected components from one or two carefully matched donors is made possible by this technique in which all other fractions are returned to the donor.

Thus far we have considered only some of the benefits that accrue from component therapy but we should recognise that many of the well-described risks persist. A recent report (Lancet, 1973) reminds us that malaria and trypanosomiasis may be transmitted to patients from donors who have been in endemic areas even though their stay has been brief. Similarly, a problem creating world-wide anxiety is the transmission of serum hepatitis by donors and the advantages of reconstituted frozen erythrocytes has already been referred to. Although component therapy will not completely remove the hazards of blood transfusion, the judicious use of specific fractions may well contribute a further reduction in the hazards which attend this form of therapy.

Component therapy is now firmly established in all major centres in the world and it may be concluded that this practice has emerged as clearly superior to non-selective infusion of whole blood. In the light of increasing familiarity with the benefits that may be expected from component therapy there seems little justification for the continued use of whole blood in situations where specific fractions are obviously clearly indicated. The time is now appropriate for medical schools and teaching hospitals to critically reassess their transfusion practices and, in association with the respective transfusion services, to implement component therapy as a more economical, rational approach to the specific needs of the individual patient.

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The Social Basis of Illness

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The topic is spongy. Very little research has been carried out on the causal links between social structure and illness. But the meagre work which exists tends to indicate the existence of positive links though the mode of the link still remains enigmatic. A search in literature suggests two broad causal categories: the first is inbuilt in the society, the second results from the strains of social change. Instances of the second resemble those observed by Professor M. Gelfand. In a paper to the conference on Human Biology of Environmental Change held in Blantyre in 1971, he wrote:

"Whilst it is easy to attribute the rise in coronary thrombosis among urban Africans to their greater consumption of animal fats, eggs, bacon, etc., one sees many instances of Africans living in rural areas enjoying a very rich diet not succumbing to this disorder."

Professor Gelfand equates the differential response to changes in the social environment. He writes: "The peculiar stresses of urban life could easily be a factor in leading to a coronary attack. Indeed we know that stress can be an exacting factor in precipitating an attack on coronary occlusion. The occasional cases of thyrotoxicosis or ulcerative colitis, now being observed in Rhodesia, are ascribed to the stresses of a new environment". (Vorster 1972, page 66). Earlier work by White working in Bulawayo supports this. His observation links
REPORT OF THE REMUNERATION COMMISSION

To the Editor: Now that the Association has made an official announcement in regard to the Report of the Fourth Remuneration Commission (1974) and has quoted the Minister's replies to questions in the House, one feels free to point out some obvious inferences and possibly clear up a few misconceptions that may have arisen in the minds of those who have taken the trouble to study the bulky report. There may even be some who have read the review of some selected passages from the report that appeared in the Afrikaans press on or about 12 December 1974. This latter article, by an anonymous 'kamer', is nothing more than an inspired attempt to justify the methods and conclusions of the Commission and to place the medical and dental professions and their representatives on the Commission in a poor light. It also contains some glaring misconceptions. It would not be difficult to guess its origin.

It can be deduced from the Minister's replies that the report was not unanimous, because the representative of the medical profession did not find himself in agreement with the decision of the Commission (majority) and that he drew up a dissentient (minority) report which was found unacceptable by the Commission (majority) and was therefore not presented as a report or part of the report.

The Act itself provides for a report and makes no mention of a minority report. The report is written by the Chairman and is adopted by a majority vote.

There is a statement in the report (para. 11.38) that the representative of the medical practitioners decided to submit a dissentient report (on 16 September 1974) even before there had been a proposal or a final decision on the amendment of the fees of any of the disciplines. The inference, one imagines, is that if he had held his horses he might have fared better in the matter of increases offered.

Anyone who has studied the Act and the workings of past commissions will know that the Commission is appointed on 1 July and is required to present the Minister with a completed report on 30 September. It is a fair inference that a Commissioner who has been in attendance at all meetings—during the hearing of oral evidence and when that evidence, and the data that would be relied upon to arrive at any given conclusion, were discussed — would by the middle of the third month of the Commission's sitting, have a good indication of which way the wind was blowing. It is also a fair inference that if he saw no possibility of substantial agreement with other members of the Commission he would, at a certain point, in time, have decided to submit his own conclusions in the form of a report. To be fit for the Ministerial Desk, such a report surely needed some time for preparation and presentation in a suitable form, and the deadline would then presumably not be 30 September (in this case a Monday), but several working days earlier, say 23 September — which hardly allows abundant time for a well-written and finished article.

It is, furthermore, obvious that only a starry-eyed optimist would embark on a dissentient report without reasonable confidence that it would be accepted as such, even if it had no weight in law but merely represented a point of view. The presentation of a minority report is after all an accepted procedure in our conduct of public affairs. In the event, the Commission, by majority vote, rejected the report and it was still-born, although, as we know from the Minister's reply, it was submitted to him as a document for perusal. He is not bound to make the contents public, and the compiler is consequently also restricted from doing so. One would think that the bulky report of the majority would be able to withstand the onslaught of a minuscule minority report, but the majority of the Commission evidently considered their decision to be the better part of their valour.

An informed criticism of the report of the Remuneration Commission will appear in the SAMJ over the name of the General Secretary of MASA, and there is no one better equipped to deal with this unfortunate document.

For 'representative of the medical profession' read the undersigned.

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WHOLE BLOOD OR COMPONENTS — A NATIONAL PROBLEM?

To the Editor: Whole blood is the traditional way of correcting anaemia, and any deviation from this well-established practice requires careful evaluation before it can become an accepted alternative. It is therefore appropriate that the question of component therapy should be critically examined in the light of an increasing demand upon the limited supply of blood available in this country. A prime consideration is the fact that a single unit of whole blood contains many fractions which, if separated and then used selectively, will adequately correct anaemia, provide components which can be used rationally to treat a variety of other deficiencies and, equally important, reduce at least one risk associated with blood transfusion — serum hepatitis.

Many difficulties attend the use of whole blood, including the belief that it is physiological. It is important to appreciate that a low pH and a potassium concentration which rise progressively with storage, create a situation which is far from physiological, and this becomes positively dangerous as the volume of whole blood infused rises. Similarly, the belief that whole blood is the most appropriate way for reseeding clotting factors is also incorrect, since the balance between red cells and plasma elements is grossly disproportionate. Undoubtedly, indications for the use of fresh whole blood do exist, but the rational use of components would go a long way towards restricting irresponsible requests for whole blood — an important consideration regarding the special blood typing policy necessary to provide fresh blood. Thus, with regard to the red cell concentrates, there is no longer any doubt that this is the component of choice for the treatment of patients with reduced oxygen-carrying capacity and normal blood volume. When there is associated reduction in blood volume, the packed red cells are conveniently combined with a crystalloid, such as saline — a practice clearly shown to be as effective as transfusing whole blood in those situations where the initial haemoglobin is approximately 10 g/100 ml and total blood loss is less than 2 000 ml. An additional benefit is the fact that long-term storage at low temperature is becoming a practical way of red-cell preservation with oxygen-carrying capacity maintained intact. While the initial cost of such a programme is somewhat higher than that of fresh packed cells, the advantages must be measured in terms of availability of rare blood groups and the ability to balance a varying supply and demand. Any such system must take into account the special advantages offered by newer anticoagulants and additive solutions designed to promote improved shelf-life of packed red cells.
The provision of platelets is a further excellent example of the benefits that a properly structured component therapy programme may offer. High concentrations may be administered at relatively little volume, and stored platelets may retain significant activity for up to 72 hours under carefully controlled conditions, although some 4 hours are required in the circulator for platelet function to return to normal. The increasing dependence of clinicians upon platelet concentrates for intensive haematological support underlines the need for prospective, controlled research programmes of those centres where, for example, aggressive cytotoxic chemotherapy programmes are in operation for the treatment of patients with acute leukaemia.

It is important to recognize that platelets are maximally effective in situations where production is defective and unlikely to achieve significant improvement where antithymocyte globulin or chemotherapy is in use. Furthermore, the assessment of biological activity of the infused platelets is vital and depends upon a precise knowledge of platelet life-span and the characterization of the patient's haemostatic response to therapy, and an inflexible quality control programme offered by the transfusion service, including the definition of such things as platelet survival in normal and abnormal recipients.

Repeated platelet infusions may be associated with shortened survival on the basis of isoantibody production, and in these situations additional benefits will accrue from the use of HL-A matched platelets. Such an approach places an additional load on the transfusion service, but there can be no doubt that such a challenge must be accepted when all available evidence indicates an increasing need for greater sophistication in the production and use of platelets.

In much the same way, granulocytes are now indispensable to the treatment of drug-induced bone-marrow failure, with its associated granulocytopenia. There can be no doubt that this component will be increasingly required in haematology and oncology. The recognition that patients with granulocytopenia who have obtained yields of functionally intact granulocytes may be obtained by different techniques, including continuous-flow blood separation. This aspect of component therapy is somewhat limited, since granulocytes do not readily lend themselves to storage, and it seems prudent, at this time, to restrict their production and use to specialized units committed to the care of patients with haematological malignancies and equipped with the necessary machinery and expertise for their production and objective evaluation. Furthermore, the likelihood of obtaining, and HL-A matching, is very largely restricted to sibling donation, while high yields from patients with chronic granulocytic leukaemia will, of necessity, be available only in the special setting of a leukaemia centre.

Among the many other components that are derived from the plasma, which include cryoprecipitate, a variety of hyperimmune sera, and a selection of high-potency clotting factor concentrates, including fibrinogen. All these are now in standard production by modern transfusion services, and there is no doubt that their use provides the best available means for selective replacement therapy in the haematologically unbalanced patient.

It is clear from these arguments that compelling reasons exist for providing components which can be dispensed by the blood banks in much the same way as a pharmacist provides individual products on specific indication. The amount of blood in this country is limited and the demand is increasing steadily, so that it is incumbent upon the medical profession to make the best possible use of this precious commodity, and it is self-evident that a properly structured component programme provides the one practical way of achieving this highly desirable objective. There are certain implications for such a suggestion, in that university and hospital staff must acknowledge the shortage of blood, they must recognize the need for a component programme as a responsible means of meeting this difficulty, and thus implement teaching at undergraduate levels and encourage it actively at the practical postgraduate level. In turn, the transfusion services must gear themselves to the provision of components, must accept the full responsibility for the introduction of properly designed quality control programmes, and must ensure that the products offered are dependable and in adequate stock. In the interests of national unity, the question of centralisation of much of the component programme has much to recommend it, and serious consideration needs to be given to the establishment of a single centre which will provide an environment suitable for research into all related aspects of blood transfusion, perhaps coupled with satellite regional services geared for distribution and the provision of specific needs less well suited to centralisation. Urgent thought needs to be given to the provision of such a facility before it is too late, and an opportunity for realistic consolidation is lost forever.

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METABOLIC ACIDOSIS IN THE ACUTE ABDOMEN

To the Editor: The article by Mendelow and Miency1 was of interest.

In group I, patients with mesenteric vascular occlusion, 3 patients were shocked and had base deficits of 13, 21 and 22. One patient was not shocked and had a base deficit of 4. No other patient in the study was shocked. Statistical comparison between the base deficit of the shocked patients and the control group (group IV) using a non-paired r test revealed r = 5.5; N = 6; P = 0.0075. The assumption must thus be made that shock, and probably dehydration, from whatever the cause, were the prime movers in the production of a basic deficit.

It is known that hypoxia produces a secondary lactic acidosis2 and it may well be of interest to study lactic acid concentrations in patients with infarcted bowel. If the results of such a study were significant, lactic acid levels could still be used as a clinical diagnostic aid, since the assay takes only 1 hour, and could probably be accelerated.

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DRUGS IN OBESITY

To the Editor: There have been a few comments on certain statements in my review on 'Drugs in obesity.' The facts are based on reports in the literature cited in the 20 references given in the article. Far those interested in weighing the pros and cons of fenfluramine and mazindol, there is a concise balanced statement in the Drugs and Therapeutics Bulletin (1974), volume 12, page 101.

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THE TECHNICAL ASPECTS OF CRYOPRESERVATION OF HAEMATOPOIETIC STEM AND PROGENITOR (CFUc) CELLS

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The capacity of haematopoietic stem and committed progenitor (CFUc) cells to withstand long-term low-temperature storage, whilst retaining their viability, has considerable potential in experimental and clinical medicine. The establishment of dependable techniques for cryopreservation will provide a practical means for autograft rescue of patients using their own bone marrow and therefore freedom from transplantation complications such as rejection and graft-versus-host disease. The individuals most likely to benefit from such a technique are those with haematologic and non-haematologic malignancy where myelotoxicity is the factor limiting the delivery of radiotherapy or cytotoxic drugs in doses aimed at disease eradication. Bone marrow is collected under sterile conditions in thromboliquine. It has been established that freezing of the entire buffy layer is unsatisfactory due to a high cell loss consequent upon post-freeze agglutination. The cells are separated on a ficoll-hypaque density gradient using a modification of Boyum's method in which the marrow is diluted in tissue culture medium containing foetal calf serum rather than in a protein-free balanced salt solution. An improved recovery of CFUc has been documented. The cells are frozen in a final concentration of 10% dimethylsulfoxide and the 1% remaining at the time of tissue culture is non-toxic to CFUc. The freezing chamber is pre-cooled and after loading with the samples temperature is reduced at a rate of 10°C per minute. This point is critical and recorded using a carefully insulated probe in one of the actual samples. Cell viability is improved by anticipating the temperature rise associated with the latent heat of fusion and no plateau is allowed to develop at this point. The reconstitution of cells from the liquid phase of nitrogen storage is adversely influenced by failure to rapidly thaw the specimen from -196 and from this point to reconstitute slowly with tissue culture medium. The viability of cells is characterised by in vitro bone marrow culture and in vivo capacity for bone marrow repopulation potential using an animal model.
PERSPECTIVES IN BONE MARROW TRANSPLANTATION

To the Editor: The establishment of an active human bone marrow transplantation programme at Groote Schuur Hospital, as a participant in the international aplastic anaemia collaborative study group, has indicated the need to place in perspective the anticipated benefits and the recognised limitations of this procedure.

The technique is not new, but until recently it has enjoyed only limited application in clinical medicine, largely because the earlier attempts had an unacceptably high morbidity and mortality. However, results reported in the past 5 years are much more encouraging; these undoubtedly reflect an awareness of the place of meticulous tissue typing in donor selection, the availability of protected environments, and an increasing use of all-antigenic granulocyte support. Other factors which contribute to the improved success rate include better methods for the control of rejection and graft-versus-host disease coupled with more effective therapy for the infections that are particularly complex in the immunocompromised host.

For these reasons it is appropriate to review the place that this procedure occupies in selected clinical situations, especially in the management of patients with aplastic anaemia and those with refractory haematological malignancies.

Bone marrow transplantation is not to be undertaken lightly and it is mandatory to recognize and accept the wide-ranging commitment that is an integral part of the successful and responsibly conducted programme. Thus, at laboratory level, reliable facilities are required for meticulous tissue typing and the performance of mixed lymphocyte cultures, upon which proper donor selection rests. Similarly, it is essential that all the necessary methods are available to characterize each graft in terms of cellularity, viability and stem cell content, the last monitored by colony growth in soft agar. In this regard, the first human bone marrow transplant in the Western Cape was performed more than 2 years' experimental work in animals to allow for the adequate development and standardization of these procedures.

Similarly, no programme should be initiated at clinical level without full access to a protected environment, intensive care nursing facilities of the highest order, and a thorough familiarity with the drugs that need to be used. The surgical technique must be accurately controlled, and the whole programme revolves around an active cell support section to provide properly matched and irradiated granulocytes and platelets during the post-transplant period and should be readily available. To retain a high degree of flexibility, we operate both the NC1/IBM continuous flow blood fraction separator and the Fahnert blood separator; in selected situations bone marrow reconstitution is a multidisciplinary undertaking which involves microbiology, respiratory pathology, psychiatry and gastro-enterology, to mention but a few associated groups.

The Cape Town bone marrow transplant programme was initially entered upon to provide definitive treatment for individuals with aplastic anaemia; of the 14 patients admitted during 1972, 13 died and the median survival was under 4 months. There is little reasonable doubt that aplastic anaemia, which must be clearly distinguished from varying degrees of bone marrow hypoplasia, is critically dependent upon marrow reconstitution, since other forms of therapy have been shown to be of very limited benefit to these patients. On the basis of available data, the use of an identical twin as a donor is associated with a success rate of approximately 50%. HLA-identical and MLC non-reactive siblings make possible engraftment in a similar percentage of recipients, with survival approached 50% in some. Observations extend from 4 to 40 months. It must be emphasised that while rejection, infection and graft-versus-host disease continue to challenge the investigators, the greatly improved survival underlines the importance of improving, in concert with the development of this therapeutic modality.

A second group of individuals likely to benefit from the availability of bone marrow transplantation are those with acute leukaemia in whom conventional forms of therapy have been unsuccessful. Patients with refractory tumours, where a severe degree of bone marrow depression with residual disease are obviously unsuitable for further cytotoxic chemotherapy. In this situation aggressive cytoreductive chemotherapy, followed by whole-body irradiation and transplantation, offers a new dimension in treatment. The Seattle group has reported that, with a suitable donor, approximately 25% of these patients in this group achieve complete remission. The magnitude of this particular problem is not, as yet, defined in either the Western Cape or in the Republic of South Africa.

The question of bone marrow transplantation in patients with non-identical bone marrow donation has now emerged as a major challenge for groups dedicated to the study of this disease.

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Die Oorplanting van Beenmurg

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Opsomming

Beenmurgoorplanting het gevestig geraak as die voorkeurbehandeling vir pasiënte met ernstige akute aplastiese anemie of immuuntekortsiets. Indien 'n geskikte donor beskikbaar is, is in hierdie omstandighede vroeë verwysing, voordat isolimmunisering volg op bloedtransfusies, granulosielwat- of plaatjes, tot die grootste voordeel van die pasiënt. Konservatiewe versorging met anabole en androgene by aplastiese anemie blyk oneffektief te wees en behoort dus vermy te word.

By die leukemieëse pasiënt kan allogeneiese oorplanting gebruik word vir die eindstadium van die siekte; maar die herstelkoers is teen beste tussen 10 en 15%. Onlangsse gegewens bevestig die aandeel van hierdie proce- dure by die eerste volledige remissie by volwassenes met leukemie en soortgelyk by kinders waar swak prognostiese tekens aanwezig is. Die eindrol van beenmurgoorplanting in hierdie omstandighede moet nog opge- geklaar word.


Bykomende immunologi- see metodes sal ontwikkel moet word vir die uitkenning en daaropvolgende verwydering van be- smettende tumoroselle van die ent voor sy herin- fuus. Beenmurgoorplanting is dus 'n duidelijk gevestigde terapeutiese prozedure. Alhoewel nog in die vroeë fase van ontwikkeling is duidelike aanduidings omskryf en is 'n vaste toewydig nou tans nodig om die plaslike deskundigheid te ontwikkel asook geneewes om die toenemende aantal verwysde pasiënte te onderskep.

Summary

Bone marrow transplantation is established as the preferred form of treatment for patients with severe acute aplastic anemia or immunodeficiency diseases. In these situations, provided a suitable donor is available, early referral before isolation has resulted from transfusion of blood, granulocytes or platelets in the patients' best interest. Conservative management with anabolic androgens in aplastic anaemia has been shown to be ineffective and should therefore be avoided.

In the leukaemic patient allogeneic transplantation can be used for end stage disease but the salvage rate is at best between 10 and 15%. Recent data have established the place of this procedure in first complete remission for adults with leukaemia and, similarly for children, where bad prognostic signs are demonstrable. The final role of bone marrow transplantation in these situations remains to be clarified.

should decrease with improving general education and early re- ferral of patients. Secondly, graft versus host disease is frequent and poorly understood complication. This unique immunologic phenomenon places limitations on the use of transplantation and presents investigators with their greatest current research challenge.

Autologus bone marrow transplantation has neither of these complications. It however presents a formidable investment of time in technology necessary for the collection, concentration and cryopreservation of haematopoietic stem cells. Additionally immunologic methods will need to be developed for the recognition and subsequent removal of contaminating tumour cells from the graft before its reinfusion. It is concluded that bone marrow transplantation is a clearly established therapeutic procedure. Although still in its early phases of development clear indications have been defined and a firm commitment is now necessary to develop local expertise and facilities to meet increasing patient referral.

Die oorplanting van hematopoetiese stamselle uit die beenmurg van een persoon na die van 'n ander het sy regmatige plek ingeneem by die goedgevestigde, alhoewel nog ontwikkelende, oorplantings- tegnieke beskikbaar in die moderne mediese praktiek. Dit word aanvaar dat die ernstige akute aplastiese anemie of by immuunetekortsietsie dit nou die behandeling van keuse is met statisties duidelike voordeel bo die van konservatiewe behandeling. Minder duidelikheid bestaan wanneer leukemie, Fanconi-anemie of sommige van die lateverlopende genetiese siekte soos homosigoti- se talassemie oorweeg word. Dit is 'n groot mate hang hierdie beperking, in die omvang van beenmurgoorplanting, saam met die gepaarde gane en sterfte te wye aan die onvermoë om implantering te bewerkstellig, aan hul diegene en aan die unieke immunologi- se verskynsel van ent-teen-gasheer-siekte. Laasge- noemde, met die paradox dat dit slegs
by geslaagde inplanting voorkomt, kan tot bij 60% van de pasiente voorkomen en bovendien dodelijk verloop. Om de huidige aandeel van beenmurgoorplanting in perspectief te stellen, is dit nuttig om sommige van de studies op diepe en in

tabel I: Aanwijzingen van beenmurgoorplanting

Geestig


Ontlukende


Betuiebaar

Dodelike genetiese skies en konstitutionele anosciëne.

TABLE II: Komplikasies van Beenmurgoorplanting

Vooroorplanting


Intraoperatief

Skenergevaar. Pasiëntgevaar.

Na-oorplanting


Die komplikasies vóór oorplanting kan tot 'n minimum beperk word deur die opleiding van die huisarts en vroeër verwysing van die pasiente.

Tydens oorplanting is die skener in gevaar vanweë die algemene markse en ook volgende op die meervoudige naaipuntes vir die ver- krywing van die beenmurg. Die gevaar vir die pasiente is klein; dit is die gevolg van 'n tyde- like daling in okusgering volgende op die ent-infus.

In die periode na oorplanting is dit gebiedend dat voldoende skelaan beskikbaar is om bloeding en infekkie te behandel. In hierdie tyd neem verwerping toe direk in verhouding tot die aantal voorafgaande bloedtransfusies. Ent-teen-gasheer-siekte is onvoorspelbaar en dus die onderwerp van navorsing.

Die laboratorium met hul tegnieke procedures en resultate in oënsku te neem.

Dierestudies

Die moontlikheid van beenmurgoor- planting is meer as 25 jaar geleide gestel met die aantoon dat die hematopoe- tie se heerlikheid wel plaasvind by le- tantalbestande knaagdie indien die milt van die straalveld beskerm is, of indien murg intravenerus ingeborg is. Kliniese studies is bevorder toe Mathie e.m. oor- plantings gedaan het op 'n aantal pas- inte wat aplasties geword het g.e.v. 'n onwel met 'n kernreaktor. Sederdien is hierdie procedures meermale aangedurf en is die gegevens deur Botin' sistematies ontleed. Die betreklik swak resultate wat tot op daardie tydspan verkry is, toon die swak instig oor die belangrikheid van weefselstaping. Oor die afgelope 5 jaar het bekend geword dat meer as 100 persoon selle hematopoiëtiese funksie kon handhaaf vir meer as 'n jaar van langer na beenmurgoorplanting. Hierdie stand- houende verbeurting is te wye aan beter passing tussen skener en ont- vanger.

Dit was deur die gebruik van inge- tekte dierstamme, wat dieselfde weefsel- antige deel en dus geen immunologi- seke skies tot oorplanting of geënt, wat die modelle verskaf is om 'n aantal belang- rike oorplantingsbeginne te omskryf. Hulle bevat: die minimum aantal selle benodig vir geslaagde oorplanting, die handgrepe benodig om oorbliveende immuunfunktie by die ontvanger te onder- druk en sodoende oorplanting meer uni- form te maak, en die moontlikheid dat immunologieskompetente selle van die skener ook antitumorwerking na die gasheer kan oordra.

Die diermodel is het ook 'n belangrike rol gehad in die onderzoek van ent-teen- gasheer-siekte waarin ontstekingsreakties gerig is teen lewer, vel, en maagdiemka- naal deur selle van die nuutgevestigde oorplanting. Met die meganisme nog onbekend word gemaan dat limfosiëte en makrofage in die patogenese betrokke is en KAY7 die belangrikheid bekleen- toon van antigenie-dispariteit, infektie, en voorafgaande geneesmiddelbeurting of besturings om die felheid van hierdie ver- skynsels te verhoog.

Dierestudies het verder getoon dat in- feksies nie maklik onderskei kan word van ent-teen-gasheer-siekte nie (Jones e.m.), daar sowel die voorokse as die felheid verminder is wanneer muis 'n klein vrye omgewing onderhoud word. 3, 16, 11 Die daartussen van 'n soortgelyke on- standigheid by die mens is 'n geweldige groter uitdaging en dit is te betwyfel of volledige vrywaring teen besmetting deur mense moontlik is.

Proefdierroutines i.v.m. die beheer van ent-teen-gasheer-siekte is bekend. By beenmurgoorplanting is daar die oordrag van hematopoëtie se stamsele saam met 'n aantal immunologieskompetente lim- fosiëte na die gasheer; in daardie omstandig- heid waar laagvemde tolerant is teenoor antigene van die ontvanger, 2008 by singeneesi oorplanting, kom ent- teen-gasheer-siekte nie voor nie. Die allo- geniese oorplanting is oor die meer ge- bruiklike en hierby is pogings minder geslaag om die ontstaan van die uitbreiding te voorkom, of die felheid daarvan indien geestig te verminder. Studies is teens daaropmerking waarin gepoog word om die verweterende werking van immunologi- sekompetente selle te wysig, bepaal in dierroutines is sowel parenterale Coryne- bacterium parvum as SIKLOPOOR- A3 doeltreffend. Dit moet nog vasgestel word of die gebruik van hierdie immunolo- giese adjuvante by die mens 'n soortgelyk effekt kan bereik.

Laboratoriumstudies

Een van die hooflikers wat die doel- treffendheid van die ont bepaal, is die
verskaffing van 'n voldoende aantal hematoïde stamcellen. Terwyl die aantal kernstelle oore Gedraad dit in die algemeen kan verseker, is aangetoon dat bekamurkultuur 'n emnoude praktiese wyse is om uit te ken welke infusies minder as die benodigde aantal voorlopers bevat. Met 'n uitgestrekte tektork beheer oorweging gegee te word aan die aanvulling van die aantal stamcelle deur daglike infusie van geëepeen verkry van die skenner deur senufruger in bloedeffraktes met deurkopende vloei.13

Beemurkultuur in die laboratorium kan ook ter sake wees in die poging om pasiënte uit te ken met ernstige akute aplastiese anemie waar die letse te wyte is aan 'n defektiwe stamcel en die verwanking van hierdie bevolking nodig is. Daaromnoor is 'n aantal pasiënte tans uitgekeer waar immunologiese oppressive oorheers, en reaksies gevolg het op immunoonderdrukkende terapie waar plomptege gelaat het.16

Murgoorplanting by Mense

Pasing vir weeselsanaarbaarheid blyk te wees een van die belangrikste bepalers vir geslaagde implantatie. Die belangrikste komplices van weeselsanaarbaarheid by die mens is bepaal deur 'n reeks genes wat antagon steur op die seopepervlak kudde, sommige waarvan serologie bepaal kan word, andere gedeelte deur 'n reaksie in vreemde limfosië.17 Terwyl daar weinig twyfel bestaan dat hierdie stelsel van oorwonebare basis in die berekening van die beste pas van skenner en ontvanger, is daar nog meningsverskl onder die relatiewe belang van die twee tegnieke vir antigeneuitkenning.

Ongetwyfeld is volmaakte pas die beste manier om ontwerpvering te vermindere ('n hierdie probleem op 'n laagste voorkom al by identifiseerde tweelingoorplanting of waar ingegteelde stamme van dieprepers gevoel is. In allogenese oorplanting waar gepasse broer of suster as skenker dien, is dit noodsaaklik om die immuunfokus vir oorplanting in die pasiënt maksimaal te onderduik gewoonlik deur die gebruik van intensiewe toediening van sitorioksi. By pasiënte met leukemie bestaan die verdere probleem om die residuele skiik uit te wus sodat heellangamendelkiew dié gepas word en die medikamente as deel van die voorbereiding. Hierdie procedures bring wees 'n aansienlike graad van skiik. Baie werk word gedaan om te bepaal welke van die verskeie programvorsing die meeste immunoonderdrukkende of verdraagbare neew effekte bereik.

In bepaalde omstandighede waarin die lewensvare eentjie met die eerste poging faal, is verdere voorbereiding noodsaaklik gevolg deur heroorplanting. Die ervaring tot dusver is beperk maar by 'n aantal pasiënte was dit geslaagd.18

---

### Table 2: Die Aandag van Laboratorytngemie in Murgoorplanting

<table>
<thead>
<tr>
<th>Aandag</th>
<th>Kolonies</th>
<th>Trosse</th>
</tr>
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<tbody>
<tr>
<td>1e</td>
<td></td>
<td></td>
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<tr>
<td>2e</td>
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</tbody>
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### Fig. 2: Die Aandag van Laboratorytngemie in Murgoorplanting

Die boomsurkultuur in vitro met gebruik van 'n gestandardiseerde tegniek (Jacobs et al, 1979) is 'n nuttige laboratoriumgid in boomsurgoorplanting. Die groei in kultuur is swak by aplastiese anemie maar waar dit verbeter in die aanwesigheid van antimitosiegolulien (ATG) kan die pasiënt reageer met of sonder 'n ent. Hierdie tegniek is 'n belangrike metode om 'n ontstrekende ent van te stel wat dan aangeval kan word met 'n infusie van hematoïde stamcelle daaruit versamel deur aanbouwende vloeis en senufruger van bloedeffraktes. Waar toetrekke groei in vitro aangetoon is kan die vat van die ent verwag word.

### Tegniek van Beenmurgoorplanting

**Thomas c.m.** het na langdurige en sorgevulde werk die metodes gestandardiseer. Die ontvanger se retikubendoteelstel word aanvanklik geprikkel met antigene bekoom uit die skenner se geel laag, en voorbereiding met siklosofo mied 24 uur later begin. Hierdie gevolgeverbouingse infusie18 prikkel die kompetente limfosië tot vermeerdering wat hulle selekty gevoelig maak vir die sitoriokië verwerking van die allikheerstof.

Beemurkultuur word deur meerder aspirasies van kleinvolume uit die sterone en die leukamia versamel. Nagenoeg 500 ml van murgkybloed word stadig intraveneus aan die ontvanger toegedien. Gedurende die omstandighede 14-21 dae benodig vir implantaat, by die pasiënt swaar pasiëntoenies en benodig hulle intem ei steun met witselu en platoor. Dit word geraakaf vanaf die skenker verskaf deur deurhendue-vloeiskeile, of waar onnodig vooraf 'n standaard skenkerpanel. In die meeste oorplantingseenhede vorm die afdeling selstel 'n integrale deel van die oorplantingseenheid.

**Ydeus** hierdie pasiëntoenies tydepest is swak mikrobioloogise beheer oor eksogene organismes belangrik vanwee die cepte onderdrukking van die immuneheer van die ontvanger, wat hulle hoogvatbaar maak vir fulminerende bakte riole infeksies en die inval deur opportu nistiese organismes.

Inplanting is gewoonlik waarneembaar tussen 10e en 21e dag en kan deur beenmurgkybloed of trebenbiopsie bevestig word.

---

Met die implantaat aldis bereik, staan die pasiënt voor die volgende en miskien ernstiger gevaar nl. die ent-teens gasheerskie. Ondanks bestendige verbetering in die bepaling van weeselsanaarbaarheid en in die ontvanger se voorbereiding by die voorsom en die eres van hierdie immunologiese besonderhede onverander. In 'n prospektiewe ewekansige studie het Thomas en sy groep20 getoon dat die geslagraffmising tussen skenker en ontvanger en suggers die weerspanningheid van die gasheer tot luktlike skenkerplanet in die omstandighede van die skenkerpatroon en terwyl dit dikwels selfverwerking is, kan dit to 'n wydspreided sklerodermatose uitbrey. Tot dusver was profilaksie nie geslaag nie en is bebehandeling van die gevegste skiekie slegs ten dele geslaag.21 Van besondere betekenis is die onlangse verslag dat een-teens-gas heer-skiekie effekief is in die vermindering van die voorsom van leukemiese tegrug by pasiënte waar oorplanting vir hierdie rede onderwerp is. Onthoud egter van die algehele sterfte toon weinig voordeel vir hierdie groep omdat dit dusver pasiënte wat hierdie komplikasies ontwikkeld 'n hoër voorsom by volwassenes van kleale respiratoriese sparringsvorm toon.
Beenuurgaplasie

Die grootste probleem met die ontlen-
ding van resultaat bereik met konserwa-
tiewe behandeling teenoor die by buen-
murgoorplanting vir pasiënte met apla-
tiase anemie, is die versui in bly wer-
kers om hul kriteria duidelijk te umkryf.
Die onlangs ooreenkomstig 'n interna-
tionale saamwerkende studiegroep1 het daardie subgroep van pasiënte duidel-
lik uitgeken waar buenmurgoorplanting aansienlik beter oorlewing as ander
vorms van behandeling bied. Hierdie pa-
siënte word gerekend deur 'n plaatjie-
telling van minder as 20 x 10⁶/µL, granu-
losiie minder as 0,5 x 10⁹/L en 'n aangep-
paste retikulatoietelling van minder as 1%.

Thomas e.m.20 het by so 'n uitgesoekte
groep aangetoon dat met gebruikmaking
geneeskrachtige oorplanting dit 'n doen-
like procedure is, met drie van hul pa-
siënte nog in lewe en gesond en die
langere oorlewingstyd meer as 16 jaar.
Die verdere belang van hierdie waar-
neming is dat dit die fout in die samentew-
derstreep wat vervang kan word vanaf
' n gepaste skenker. Hierdie gegewens,
gepaard met resultate van die internatio-
naal saamwerkende studiegroep, bevesti-
g duidelijk die plek van buemurgoor-
planting by aplastiase pasiënte. Dit is
eiger nodig om te beklemtoon dat ver-
wysing onmiddellik by die diagnostie-
king gebiedend is om in vitro-sensitise-
rings soveel moontlik te verminder wat
die kans op sukses met opvolgende oor-
plantingspogings op te werk.

Van onlangs belang is die verslak25,16
dat, volgende op aggressiewe sitiatieska,
enkele pasiënte 'n hemopoietiese her-
stel sal ondervind ondanks die feit dat in
planting gefaal het. Hierdie waan-
nemings bring die vraag na vore van alter-
natiwe patogeeniese mekanisme en die
kiesing dat immunoogebiede disfunksie van hemopoietiese voorlo-
perscele, of 'n ongunstige mikrooomgewing
ween, buemurgoorplanting kan meebring.
Daar word ook op gelet dat hoe dorstings
siklofoamfie van hierdie individue, blykbaar met die residuele bloedvorm-
ende selle vernietig nie aangestig hulle
later 'n spontane herstel meegebring het.
Dit skyn dus belangrik om te bepaal
welke pasiënte met enkele buenmurgoor-
plantasie herstel benodig op die basis van
diegete samentewende teorien die
ingeenheid van die immunoogebiede
mechanisme van her-
sters.

Hierdie gegewens van geneeskrachtige
oorplanting en die risiko van samentew-
ding van hierdie pasiënte word dalk
as se foto genoem. Die resultaat van die
planting met enkele selle wat in die
ent ontstaan.

Chroniese Granuloomse Leukemie

Die gebruik van buemurgoorplanting
by hierdie pasiënte bly nog onduidelik
van aard. Alhoewel sitiatieska chemote-
rapie of melngetralief 'n tydelike beheer
bereik, is die mediane oorlewing nie we-
senlik verleng nie. Teories is dit moont-
kan ook ontstekende vleigmentering
verwek en onduidelik skiel in die vorm
van skleromaatk. Vreug uitkoms en
behandeling met kortkortkortkortkort
korttektiesproses. Die
dag word
gemeen dat in die chroniese pasiënte
van die siekte, die aanvallende limfosiite
as deel van die ontstaanste in
ontstaan.

Versoepelingsbehandeling is
behandeling in onbevredigend. In
prof-
diere is pogings om die iets van die en-
teen-gasheer-siekte te wyk nie geslaagd
t nie. (Parker et al, 1976, Jacobs et al,
1979.)
lik om sirkulerende stamcellen te versamel en hulke vir in 'n auto-entherstel te berg, na uitwissende stiotoskiese of 'n gekombineerde modaliteit van behandeling by die aanval van versamelde siekte of blas-tomvorming. Sodanige benadering bly in wees verdrag daarin dat leukemische selye in die pasiënt teruggebring word.

Van groter betekenis is die waarne-mings dat die Philadelphiachromosoomarmende kloon uitgewas kan word deur aggressiewe voorbereiding van allo-geneiese beenmurgoorplanting.28 Met bevordering van hierdie studies asook van die versuim om die neoplastiese kloon te hergrys, sal beenmurgoorplanting ver-skyn as die eerste keuse van behandeling met enige kans op sukses by pasiënt met chroniese granuloidse leukemie.

Akute Leukemie

Beennmurgoorplanting by leukemie was oorspronklik beperk tot pasiënt met 'n gapeaste broër of suster nadat die gebruiklike medicamente gebruik het. Dit is ongelukkig nie aangebied na meerdere terugvalle of waar weerspannige siekte gevestig geraak het. Selof onder sodanig hoog ongunstige omstandighede het tussen 10 en 15% van pasiënte tot langer as 2 jaar siektervry gebleek. Hierdie gereg-wens verskaf wetenskaplike bewys om die gebruik van beennmurgoorplanting by sowel volwassenes as kinders te ondersteun selfs nadat die eerste terugval gebeur het.

Tot op datum is sodanige individue nie veilig uitkenbaar nie wat as 'n verdere argument dien ten gunste daarvan om die prosedure aan te bied aan diegene waar, alles in ag geneem, die vooruitstig oungunstig is. Die uiteindelike plek van beenmurgoorplanting by hierdie pasiënte bly onbepaald en sal 'n klein aantal sentrum suggereer dat hul vermoënt moet betrek om inligting te bekom. Beheerdata sal uiteindelike beskikbaar kom in die vorm van pasiënte waarvoor ge-paste skenkers nie beskikbaar nie of by diegene wat om een of ander rede en goed ingelig, tog verkeers om beennmurgoorplanting nie te aanvaar nie.

Ootola Beennmurgoorplanting

Die ontwikkeling van tegnieke vir die berging — by lae temperature en oor laagtermyn — van sorgvuldig versamelde en uitgewasen stamcellen uit menslike beennmurg of perifere bloed, het 'n aantal nuwe en aantreklike behandeldingsmogelijkhede meegebring. Alhoewel auto-entherstel — soos bosp forasio en chroniese granuloidse leukemie —, van beskikbaar is dit waarskynlik met voldoende leukemiese blastes besit om die murg te herbeseend en nuwe siekte te verwek, is dit nie noodwendig die geval by solide tumore nie. By laaggenoemde bestaan die moontlikheid om onbesmet beennmurg van pasiënte te bekom en dit vir hervorming in 'n tydspan waar murg reorgeniëring deur radioterapie of chemoterapie 'n beperking bring op die hoeveelheid van behandeling wat met veiligheid toegedien kan word.

Weer eens sal dan die aandeel van auto-entherstel in die geneeskundige praktiek uit- eindelik pas heder word soos geegens inkom en sukses as geldige vergelykings moontlik maklik tussen resultate by pasiënte met oorplantings en pasiënte met ander vorms van behandeling.

Samevatting en Gevolgtrekkings

Beennmurgoorplanting is 'n goedgeves-tigde vorm van behandeling by pasiënte met ernstige akute aplastiese anemie en immuunpostskietes. Met die voorbe- houd dat 'n geskikte skenker beskikbaar is, is die duidelike bevordering van voorkeur by hierdie individue die onmiddel- like verwysing vir 'n beennmurgoorplanting. In die lig van beskikbare getuiskes is daar min regverdiging om behandeling in afwagting toe te dien wat slegs die moontlikheid van later toebehoors planting kan benadeel.

Die aandeel van beennmurgoorplanting by volwassenes en kinders met akute of chroniese leukemie, Fanconi-anemie, leukeatogenetiese siektes, en volwassenes, word tans intensief nagevors. By akute leukemie met terugval by die aarde van hierdie procedure aan te bring by volwassenes en kinders met oungunste.

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**Fig. 5: Resultate by Oorplanting**

A. Ernstige saamgestelde immuunpostskiekte. Min imulie enige oorlewende is vermeld onder oorplanting. Waa dons beskikbaar is kan syfers so hoog 50% gevind word.

B. Ernstige akute aplastiese anemie. Afhangende van die kriteria gebruik in die rekke vermeld, het die kontrolepasiënte nagenoeg 2 jaar oorlewing in die streek van 10%.

Gee dat vergelyk met diegene volgende op beennmurgoorplanting met geno- singssyfer nagenoeg 50%.

C. Weerspannige leukemie. Die oorlewing sonder in oorplanting is nut: dit kan 15% siektervry wees by 2 jaar.

D. Remissie-oorplanting vir akute leukemie. By volwassenes met mieloblastiese leukemie behandel met stiotoskies is wisselende oorlewing meestal in die gebied van 15% by 2 jaar vermeld.

In voorlopige gegevens waar murg-oorplanting gebruik is en waar pasiënte hul eerste volledige remissie bereik het, is die siektervry plato nagenoeg 60%.

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**Geneeskunde — June 1980**
n Krachtige anti-prostaglandien moet als frontlinie-behandeling vir die pyt en inflammasie van artritis beskou word. Aangesien daar 'n soortige korrelasie tussen anti-prostaglandien-aktiviteit en kliniese aktiviteit is, is die logieuse eerste keuse vir maksimum-beheer oor die pyt en inflammasie van artritis 'n besonder krachtige anti-prostaglandien, Froben.

Aanbevoe doses: 150 mg tot 200 mg daglik in verdeelde doses. In pasiënte met ernstige symptomte van siekte van onlangs oorsprong, of tydens akute toenames, mag die doses vermeerder word tot 300 mg daglik in verdeelde dosisse.

Aangewys in osteoartrrose, rumatoïde artritis en gewrigsverstydende spondilitis.

Froben
Krachtige anti-prostaglandien, krachtige anti-artritis
NEW GROOTE SCHUUR HOSPITAL
LECTURE THEATRE NO. 2
E-FLOOR
THURSDAY, 22nd MARCH 2007
16h00 – 17h00

THE HISTORY AND ACHIEVEMENTS OF
HAEMATOLOGY IN CAPE TOWN
-A CONFLUENCE OF IDEOLOGIES-

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Sarison Foundation Professor of Haematology
Honorary Consultant Physician
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Professor of Internal Medicine
College of Medicine – University of Nebraska Medical Centre
Professor and Head
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Incorporating
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Constantiaberg Medi-Clinic

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♦ University of Cape Town Leukaemia Centre
♦ Medical Research Council and Cancer Association of South Africa
♦ Gwendolene Moore Trust
♦ Michael Chanani, Kalimbi and M.A. Richardson Bequests
♦ Staff Research Fund (Posse, Becker and Cancer)

ACCREDITATION
♦ Centre for International Blood and Marrow Transplantation Research
♦ European Bone Marrow Transplantation Registry
♦ American National Donor Program

APPRECIATION
♦ Professor Bongani Mayasi
  > So welcome in his department
♦ Professor Sally Benatar
  > Friend and ethicist – all these many years
♦ Our patients and teachers
♦ Donors
  > White cells
  > Money

CONFLUENCE OF IDEOLOGIES
♦ Immense humility at the Honour
♦ Awe at the challenge
♦ Credit to our University – Sir Richard Loxi
♦ Jim Thompson – the discipline
♦ Stuart Saunders – the person
  > Laboratory trained
  > Clinically experienced
  > Administers skills

FELLOW STUDENTS

COMPREHENSIVE DEPARTMENT
THE HEMATOLOGIC MIGHT OF CAPE TOWN
1945 – 1965

- 11th hour – 11th day – 11th month

- The clinical giants
  - Jack Brock - nutritional anaemia
  - Hyntie Nossel - haemostasis
  - Reuben Mibashan - the organiser
  - Helen Brown - blood in medicine
  - Clarence Merkly - the complete hematologist
  - Frankie Forman - clinician supreme

FOCUS ON
HAEOMATOLOGY AND REMING DISEASES

SOUTH AFRICA IN 1965

- Jack Metz - nutritional anaemia - Johannesburg
- Tom Bethwell - iron metabolism - Johannesburg
- Francois Retief - platelets - Bloemfontein
- Harry Grieg - integrated model - Durban

WHAT WAS MISSING?
HAEMATOLOGIC MALIGNANCIES

- Leukaemia
- Myeloma
- Lymphoma

Not missing

SCATTERED UNITY

DISCIPLINED APPROACH
- NATURE AND THE CELL CYCLE -

- Conception - idea is born
- Fertilisation - reality starts
- Differentiation - unique characterisation
- Proliferation - proving it can work
- Maturation - consolidation
- Apoptosis - immortalise by achievement

HOW WE MADE IT ALL HAPPEN
- THE MULTIDISCIPLINARY TEAM -

CONCEPTION
- THE IDEA IS BORN -

- The Pasteur Institute 1948-1954
- Blaisc Laboratory
- Tom Bethell 1954-1966
  - The salutary
- Clem Finch 1967-1970
  - The Fellowships

THIS DYE IS CAST
PROFESSOR DONALL THOMAS - LAMPLIGHTER

FERTILIZATION
- THE REALITY STARTS -
1970-1979

- Short period
- Integrated
  - Laboratory
  - Clinic
- Added
  - Paramedical professionals
  - Psychiatry
- Interactive
  - Lennard Kahn - Tumour Boards
  - Ross Steely - Combined Clinics
  - Andrew Ford - Infectious Diseases

THE HAEM-TEAM IS BORN
MAGNITUDE OF THE PROBLEM IDENTIFIED

- Blood in bottles
- Beds in D3 and D4
- Single shared medical registrar
- C8 dreading by tube tilt
- Eg: manual blood count!!

IMMEDIATE ESPIRIT DE CORPS

DIFFERENTIATION
- UNIQUE CHARACTERISATION -
  1972 - 1975

- Direction or specialised function
- Aplasia
- Leukemia
  - Diagnosis standardized
  - Protocol management introduced
- Relapse a problem
- Chris Barnard as catalyst

BONE MARROW TRANSPLANTATION
- THEME FOR THE FUTURE -
PROLIFERATION
- PROVING IT CAN WORK -
1975 - 1980

- Intense academic boycott
- South African Lymphoma Study Group born
- Cell separators donated
- Coulter model S obtained

ACADEMIC ENVIRONMENT IGNITED

THE ICE MAIDEN
EXPERIMENTAL TRANSPLANTATION

- Rabbit model
- The Basle collaboration
- Chorionic assay established
- Flow cytometry introduced

ENGRAFTMENT AND REJECTION
GVHD
**Graduation to prime time**

- The clinical protocol -

- 21/02/1964 - abortive graft
- 21/02/1974 - formal programme
- External peer review
- Internal support

**Our problems are your problems**

**Burn out**

- Skin desquamation
- Deep jaundice - liver failure
- 20 litres diarrhoea a day
- Staff devastated
- Cyclosporin era
  - Jean Borel and Sanders
  - Cardiac and renal grafts better

**Intensify support**
REDEVELOP GSH

- All together in Azo
- New separators
- Redesigned laboratories
- Dedicated and specialized
  - Ward
  - Staff

INTENSE INTERNATIONAL COLLABORATION
BENIGN DISORDERS

- Definitive iron studies
  - Polymaltose synthesised
  - Proven effective
- Erythropoiesis
  - Erythropoietin
- Anaemia in elite athletes
  - Lindsey Weight
- Thrombocytopenia
  - Plasma exchange

KEEP BALANCE

MATURATION
- CONSOLIDATION -
1986 – 1996
- New approach to reverse rejection
- Herman Waldmann and Geoff Hale
- Campath in-the-bag
- Switch to collection of circulating stem cells
- Forms Prius of acute GVHD
- Started unrelated programme – Birth of SABMR

OUTCOME ANALYSIS

WIDEN THE ACHIEVEMENT
1996

- Programme stable – run itself
- Segregation of laboratory – stifling academic autonomy
- No advanced haematology capacity outside our Department
- Discrepancy exists with Stellenbosch and Tygerberg

INFLUENCE THESE DEFICIENCIES

APOPTOSIS
- IMMORTALIZE BY ACHIEVEMENT -
1996 – 2006
- Wide international consultation
- Strong local support
- Transfer all accreditation with original Haem Team
- EORTC – MRC continued full recognition
- Relocation
  - Built new facility
  - Technology and staff

UNIVERSITY STYLE
ACADEMIC CENTRE

FULLY INTEGRATED DEPARTMENT

- Clinic and apheresis unit
- Dedicated ward staff
- Specialised transplantation laboratory
- Active in European and American Registries
- Accredited by audit on results
- Investigatorship in major international study groups
- Growth points
  - Conjoint university campus
  - Fellowships – Doctoral research programmes
  - Student and nursing exchanges

SUCCESSFUL – YOU JUDGE
Indications for Allogeneic Hematopoietic Stem Cell Transplants, 2003 - Worldwide - CIBMTR -

Probability of Survival after HLA-identical Sibling Donor Transplants for AML with Myeloblastic Conditioning, 1998-2004 - by Disease Status - CIBMTR -

Probability of Survival after Transplants for Severe Aplastic Anemia, 1998-2004 - by Donor Type and Age - CIBMTR -

CONCLUSION

- Commitment to honour the heritage of 1945 - 1965
- Harness all available resources
- The focus remains community needs
- Challenge to start transplantation - all ages
- Insurmountability of clinic and laboratory
- All staff equivalent - Professor to Janitor - Haem team
- Research - postgraduate - students - patients
- Safeguard our academic centres - private or state

NATURAL COALITION OF IDEOLOGIES
MEDICAL PROGRESS

BONE-MARROW TRANSPLANTATION

E. DONNALL THOMAS, M.D., RAINER STORR, M.D., REGINALD A. CLIFT, F.I.M.L.T.,
ALEXANDER FEFER, M.D., F. LEONARD JOHNSON, M.B., B.S., PAUL E. NEIMAN, M.D.,
KENNETH G. LERNER, M.D., HAROLD GLUCKSBERG, M.D., AND C. DEAN BUCKNER, M.D.

THE modern era of bone-marrow transplantation was ushered in by the experiments of Jacobsen, Lorenz and their colleagues, who showed that mice could be protected against otherwise lethal irradiation by shielding of the spleen or by intravenous infusion of marrow. At first it was thought that this protective effect was due to a humoral factor. By 1956, however, several laboratories, using a variety of blood genetic markers, demonstrated that the protective effect against lethal irradiation was due to the colonization of the recipient marrow by donor cells.

An article on clinical marrow transplantation that appeared in this journal showed that large amounts of marrow could be infused intravenously with safety, and described a transient marrow graft in man. It also provided estimates of the number of marrow cells needed and pointed out the potential application of marrow grafting to radiation accident victims, to the therapy of leukemia and to the therapy of patients with immunologic deficiency disorders (at that time collected under the heading of agammaglobulinemia). In the following year, Maché et al. attempted the dramatic treatment, by marrow transplantation, of six human victims of an irradiation accident. Despite the promising potential usefulness of marrow transplantation, the next decade was one of frustration and disappointment. Most marrow grafts were carried out in terminally ill patients who did not live long enough for a graft to be evaluated. The few successful allogeneic grafts were followed by a lethal immunologic reaction of the graft against the host. Recent advances in the knowledge of histocompatibility typing, in the prevention and management of graft-versus-host disease and in supportive measures for patients with no marrow function have renewed interest in the subject of marrow transplantation. In this article we shall attempt to review the basic immunobiology relating to and derived from the field of marrow transplantation and to describe the clinical progress that has been made, recognizing that this is only the beginning of a new phase in the development of a medical therapy.

EXPERIMENTAL BACKGROUND

Studies in Rodents

The availability of inbred strains of mice has made possible extensive study of the genetic systems that govern acceptance or rejection of a tissue graft. The most widely studied immunosuppressive regimens to prepare the recipient for allogeneic marrow engraftment in rodents have been total-body irradiation or cyclophosphamide (or both), with occasional addition of antilymphocyte serum. The amount of radiation required to permit engraftment and prevent rejection of incompatible marrow is higher than that which will induce fatal marrow aplasia. Total-body irradiation is most immunosuppressive if administered within 24 hours before the antigen, regardless of the nature of the antigen. Cyclophosphamide is an effective immunosuppressant in nonlethal doses, but is more suppressive if administered one day after exposure to tissue antigens of the marrow donor. Although cyclophosphamide can suppress an established cell-mediated immune response against some

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antigens in some animal models.\textsuperscript{18} It did not permit marrow engraftment in a rodent host presensitized to donor antigens several days before the cyclophosphamide administration and marrow infusion.\textsuperscript{19}

Graft-versus-host disease occurs whenever marrow or other tissue containing immunologically competent allogeneic cells is infused into a host who is unable to reject the infused donor cells. The pathophysiology remains unclear,\textsuperscript{20} especially the contribution of the host to the disease. The effector cell mediating graft-versus-host disease is probably a T cell, but involvement of other cells populations is likely.\textsuperscript{21} Although rodents with graft-versus-host disease die of infection that is presumably due to the immunoincompetence caused by the disease,\textsuperscript{22} it is possible that infection is an intrinsic part of the graft-versus-host disease process with activation of latent infectious agents.

The treatment of graft-versus-host disease is a formidable problem. Methotrexate has been effective prophylactically.\textsuperscript{23,24} Cyclophosphamide, procarbazine, the combination of cyclophosphamide and procarbazine, and cyclophosphamide and arabinoside are somewhat effective even against established graft-versus-host disease in some rodents models.\textsuperscript{25-28} Other approaches reported to have some effect in some models included administration of host lymphoid cells and antibody directed against donor isoantigens or against host isoantigens (as a form of enhancement).\textsuperscript{29,30} To date, the most impressive therapeutic results have been reported in mice kept in a germ-free environment and prepared with lethal total-body irradiation.\textsuperscript{31,32} When the animals were given allogeneic marrow the rate of fatal graft-versus-host disease markedly decreased, but spleen cells still resulted in fatal graft-versus-host disease. Finally, the literature is replete with approaches to specific or preferential elimination of lymphoid cells capable of inducing graft-versus-host disease from donor marrow suspensions.\textsuperscript{33,34,35,36} These approaches remain highly experimental and variably efficacious.

The application of marrow transplantation to tumor therapy received early attention on the basis of the rationale that tumors, especially hematologic neoplasms, might be eradicated by lethal doses of total-body irradiation and that donor marrow would repopulate the host and prevent radiation-induced death. The attempts to use such an approach have been based largely on studies in readily manipulable rodents bearing a variety of tumors as recently reviewed.\textsuperscript{37} Interpretation of the results of syngeneic marrow transplantation in tumor-bearing mice has been influenced by several findings in the area of tumor immunology: most murine tumors possess tumor-associated antigens capable of evoking a cell-mediated as well as humoral antibody response against them; adoptively transferred lymphoid cells can inhibit tumor growth but only if the cells are immune to tumor-associated antigens, but with rare exceptions, such adoptive tumor immunotherapy, although characterized by unique antitumor specificity, can cope with only a small tumor load; and in several murine tumor models such syngeneic adoptive immunotherapy when used as an adjunct to sublethal chemotherapy can eradicate even clinically evident leukemia if the syngeneic lymphoid cells are immune to tumor-associated antigens.

Allogeneic marrow transplantation for leukemia represents a final common pathway between the problems of transplantation immunology posed by the graft and the problem of antitumor chemotherapy and immunotherapy posed by the presence of leukemia. The only additional element introduced is that of the effect of graft-versus-host disease on the leukemia and possibly the effect of the leukemia on the severity or expression of the graft-versus-host disease. Barnes et al.\textsuperscript{38} postulated that allogeneic marrow infused into leukemic mice after lethal total-body irradiation would colonize the host and would "destroy," by the action of the immunity these residual leukemic cells — and perhaps also the host. Many studies with histocompatible cells have shown that graft-versus-host disease when sufficiently severe will affect some tumors, but most mice will die of graft-versus-host disease whereas a few mice will survive the fatal disease and will be tumor free. Maté suggested the possibility that one might use the graft-versus-host disease against the tumor\textsuperscript{39} but then treat the graft-versus-host disease and save the cured host. Unfortunately it is extremely difficult to control the graft-versus-host disease once it is established, even in non-tumor-bearing mice. One approach was recently illustrated in a murine tumor model in which nonlethal subclinical graft-versus-host disease was effective against a disseminated leukemia, but the same reaction was immunotherapeutically more effective when induced by lymphoid cells from donors preimmunized against tumor-associated antigens.\textsuperscript{40}

Studies in Dogs

In our laboratory we have carried out extensive studies with dogs, as an animal model of random-bred species, for studies of principles and techniques applicable to man.\textsuperscript{41} Dogs have a major histocompatibility complex called DL-A (reviewed by Albert et al.\textsuperscript{42}). Canine families are readily available for genetic studies of transplantation antigens, and canine litters provide matched sibling pairs simulating the HLA-matched human sibling pairs. Important observations include the following:

1. Studies in dogs indicated that cyclophosphamide can be substituted for total-body irradiation to condition recipients for marrow transplantation.\textsuperscript{43}

2. Marrow can be effectively stored at low temperatures in dimethyl sulfoxide for use in marrow grafting.\textsuperscript{44}

3. Stem cells in the buffy coat from the peripheral blood are able to repopulate the irradiated marrow spaces.\textsuperscript{45} Such stem cells are absent among thoracic-duct lymphocytes.

4. The dog was the first animal in which the predictive value of in vitro histocompatibility testing for the outcome of marrow grafts was demonstrated.\textsuperscript{46} Canine littersmates matched at the major histocompatibility complex survive better than mismatched ones, but approximately 50 per cent of the animals succumb to late graft-versus-host disease.\textsuperscript{47} This result indicates that "minor" histocompatibility differences also can play a part in the development of fatal graft-versus-host disease. Such disease can be pre-
vented or reduced in severity by the prophylactic use of methotrexate after grafting. \(^\text{46}\) The drug can be discontinued approximately three months after transplantation without subsequent development of graft-versus-host disease. Antithymocyte serum is of value in treating established graft-versus-host disease.\(^\text{49}\)

5. Blood transfusions before grafting, even from a donor compatible at the major histocompatibility complex, may sensitize the intended marrow-graft recipient and make graft rejection much more likely to occur.\(^\text{50,51}\) A regimen of procarbazine and antithymocyte serum preceding total-body irradiation appears to be of value in abrogating such prior sensitization.\(^\text{52}\)

6. Long-term healthy marrow chimeras can be achieved in this random-bred species,\(^\text{53}\) with chimerism persisting for at least 10 years. These animals can be used in studies on the nature of the operational “tolerance” necessary to maintain the stable chimeric state.\(^\text{54}\)

7. Certain canine spontaneous diseases such as hemophilia, cyclic neutropenia, malignant lymphoma, leukemia and other malignant solid tumors as well as hemolytic anemia associated with congenital pyruvate kinase deficiency are valuable models to study the use of marrow grafting for the treatment of these diseases. For instance, it was possible to show that canine cyclical neutropenia is not due to a deficiency of marrow regulation, but rather to a stem-cell defect that can be corrected by marrow transplantation.\(^\text{55,56}\) In canine hemophilia, orthotopic transplantation of a normal liver into a hemophilic dog resulted in complete correction of the deficiency of factor VIII. However, there are noteworthy extrahepatic sources of factor VIII, since hepatocellular normal dogs bearing a transplanted liver from a hemophilic dog show factor VIII levels equivalent to that seen in the heterozygous state.\(^\text{57}\) Marrow grafting studies ruled out the hematopoietic and lymphoid systems as sources of factor VIII production.\(^\text{58}\)

**Studies in Nonhuman Primates**

It was thought that the monkey was the animal of choice for study of problems in marrow transplantation because of its close phylogenetic relation to man. The work of the Dutch researchers has focused on the early occurrence of fatal graft-versus-host disease in the monkey after transplants between randomly selected donor and recipient.\(^\text{59,60}\) It was believed that the monkey differed in that respect from the dog and mimicked more closely the human situation, in which rapid onset of graft-versus-host disease with fatal outcome had been reported. When dog and monkey were compared, however, severity of graft-versus-host disease, time of onset and fatality rate did not seem to be different if donor and recipient were known to differ at the major histocompatibility complex.\(^\text{61,62}\) Van Bekkum and DeVries have provided excellent descriptions of the histopathology of the lesions of graft-versus-host disease observed in the monkey.\(^\text{11}\) Other studies in primates have focused on conditioning regimens. It was observed that very high doses of cyclophosphamide are needed to obtain successful grafts of allogeneic marrow. It was also found that the limiting toxicity of the antineoplastic drug in the primate is fatal cardiac toxicity.\(^\text{63}\) The problem with the monkey model is related to the fact that the animals are scarce, expensive and breed slowly. For these reasons, studies on grafts between monkey siblings have not yet been reported. The monkey is difficult to handle during the necessary intensive-care post-grafting. Also, monkeys with spontaneous hematologic and neoplastic diseases are rarely available.

**Human Marrow Transplantation**

**Histocompatibility**

Syngeneic or isogeneic marrow grafts, as between inbred mice or between identical twins, involve donors and recipients carrying the same tissue antigens, and thus there is no immunologic barrier to transplantation. A special kind of syngeneic graft, an autologous marrow transplantation, refers to infusion of the patients' own marrow that was set aside before intensive radiation therapy or chemotherapy.

An allogeneic marrow graft involves a donor and recipient of different genetic origin within the same species. Such transplants involve moderate to severe histoincompatibility and present a bidirectional immunologic barrier to transplantation. In the first place, the recipient may react against the graft and reject it. Secondly, a problem unique to the transplantation of tissue containing immunologically competent cells, the infused marrow cells from the donor may react against the host to produce the illness known as graft-versus-host disease.

In the mouse the major histocompatibility complex, called H-2, is composed of at least two serologically detected loci, an immune recognition locus and two lymphocyte-detected loci recognized by reactivity in mixed leukocyte culture.\(^\text{64}\) In addition to the H-2 region, a number of minor histocompatibility loci have been identified. The complexity of the major histocompatibility complex in the mouse seems to be reflected in the outbred species studied, principally the dog (reviewed by Albert et al.\(^\text{65}\)), the monkey\(^\text{66}\) and man.\(^\text{67,68}\)

In man, the major histocompatibility complex involves two closely associated serologically detected loci, the first, or "A," locus, and the second, or "four," locus. Antigens determined by the serologically detected loci are recognized by cytotoxic isoantisera raised by immunization or that arise during the course of pregnancy. More than 13 first-locus and 15 second-locus antigens can now be identified, and the resulting very large numbers of haplotypes make this the most complex genetic region yet recognized in man.\(^\text{69,70}\) The term haplotype was introduced by Cepellini to indicate the products of the major histocompatibility complex in haploid form. In addition to the serologically detected loci, there is a closely associated lymphocyte-detected locus just outside the "four" locus.\(^\text{61}\) Studies of the lymphocyte-detected locus are currently under way utilizing lymphocyte-detected homoyzogous cells as identified in the progeny of related marriages. Already several lymphocyte-detected types have been identified.\(^\text{67}\)

Marrow grafts between unrelated human beings carry a
high probability of major histoincompatibility due to the complex polymorphism of the major histocompatibility complex. Within a family, however, the situation is simplified considerably, since only four haplotypes can be involved (two from each parent) and since unrecognized, closely linked loci may be expected to segregate with the recognized HL-A antigens.60 HL-A typing of the family usually permits a genetic analysis of the four haplotypes, and offspring who have inherited the same two haplotypes are referred to as "genotypically matched." The apparent match can be confirmed by nonreactivity in one-way mixed leukocyte culture. Some loss of potential donors occurs as a result of ABO incompatibility, although a few marrow grafts have succeeded despite ABO differences. The number of patients with matched siblings is quite large, particularly since many patients have more than one sibling. In practical terms, in the course of HL-A typing of 533 patients with at least one sibling, we have found 255 (48 per cent) who had HL-A matched siblings.

With a large "bank" of individuals of known HL-A phenotype, it is possible to identify unrelated individuals who are phenotypically HL-A matched, some of whom will not react in mixed leukocyte culture.60 However, in our laboratory 28 such pairs have reacted in mixed leukocyte culture, indicating that a strong incompatibility still exists. The current research on lymphocyte-detected typing, mentioned above, offers a potential solution to this problem.

Preparation of the Recipient

The recipient of a syngeneic graft requires no immunosuppressive preparation. Similarly, the patient with severe combined immunologic deficiency disease requires no immunosuppressive preparation because of the nature of his disease. All other recipients of marrow grafts must have some form of immunosuppressive preparation so that they will not reject the graft. The type of preparation is influenced by the nature of the underlying disease.

In marrow grafting for non-malignant conditions, the preparation of the recipient can be directed solely at the problem of immunosuppression without concern for the problem of eradicating malignant cells. Most of our patients with aplastic anemia have been prepared with a modification of the regimen of Santos, which involves the administration of 50 mg of cyclophosphamide per kilogram on each of four days followed 36 hours later by the marrow infusion. Cyclophosphamide is administered 24 hours after infusion of antigen from the prospective donor, usually in the form of mononuclear cells.46,72 Nausea and vomiting accompanying administration are usually severe but transient. A high urine flow must be maintained to avoid severe cystitis due to cyclophosphamide metabolites excreted in the urine. The use of diuretics is recommended because of an antidiuretic effect of the drug.51,73 Since in aplastic anemia marrow "space" is already available, Amiel et al.74 conditioned seven patients with the disorder for allogeneic marrow grafting by injection of horse anti-lymphocyte serum. In three patients they observed engraftment of erythroid marrow, but not lymphoid cells, although the grafts generally were transient.

In malignant disorders, specifically acute leukemia, preparation of the recipient must involve not only immunosuppression but also therapy designed to kill all or nearly all of the leukemic cells. Total-body irradiation has been the most common means of conditioning a marrow-graft recipient. Studies in dogs showed that a midline tissue exposure of 500 rad of total-body irradiation, although lethal, was not sufficiently immunosuppressive to permit successful grafting of allogeneic marrow.64 Consistent and sustained engraftment of allogeneic marrow in the dog was achieved only when the irradiation was raised to 950 rad.61 Canine radiation chimeras have always been "complete" chimeras — i.e., analyses of karyotypes of cells in peripheral blood, marrow and lymph nodes up to eight years after marrow grafting have consistently shown only cells with donor karyotype61 — whereas dogs prepared with cyclophosphamide were "mixed" chimeras — i.e., both donor and host cells were present.49 The finding of persisting host cells indicates that cyclophosphamide may be less desirable than total-body irradiation in efforts to treat hemopoietic neoplasias by marrow grafting.

In preparing leukemic patients for grafting, we have routinely administered a 1000-rad midline tissue dose of total-body irradiation. Opposing 60Co sources are used in an effort to achieve more homogenous irradiation.79 Rotating the patient to achieve homogeneity not only is cumbersome but may be undesirable when one is dealing with a migrating population of malignant cells (leukemia). Acceptable homogeneity might be achieved with a single very high energy source but successful marrow grafts in large animals have not been reported with those technics. The acute nonmarrow toxicity is generally well tolerated, consisting of low-grade fever, immediate but transient nausea, vomiting and diarrhea and tender swelling of the parotid gland that resolves within 24 to 48 hours.76

Leukemic cells are sensitive to irradiation; irradiation penetrates to all "privileged" sites and is an effective immunosuppressive agent. However, when we used irradiation only, we observed a high rate of recurrent leukemia. To increase the leukemic-cell kill we decided to give cyclophosphamide, 60 mg per kilogram, five and six days before irradiation. The result has been a striking decrease in the rate of recurrent leukemia. In the present series of patients, we are administering additional antileukemic therapy just before the administration of cyclophosphamide and irradiation with the intent of obtaining maximum leukemic cytoreduction within the range of tolerable nonmarrow toxicity.

Graw et al.77 prepared patients with acute leukemia for engraftment by the administration of 45 mg of cyclophosphamide per kilogram given on each of four successive days. They observed early recurrence of leukemia. Santos et al.78 prepared leukemic patients with 50 mg of cyclophosphamide per kilogram given on each of four days. They observed the complete disappearance of host-type cells in the first few weeks after grafting. Unfortunately, most of their patients did not live long enough for complete evaluation, but one patient did have a remission of 11 months' duration before relapse with host-type leukemic cells. More recently, Graw and his colleagues79 have
prepared four leukemic patients for engraftment with a four-day drug regimen involving high doses of cyclophosphamide, cytosine arabinoside, 6-thioguanine and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). One of their patients with acute myelogenous leukemia is still in complete remission with a successful graft more than two years after grafting, suggesting that it may be possible to eradicate a leukemic cell population with drugs without the use of irradiation. Gengozian et al. prepared two patients with acute myelogenous leukemia with antithymocyte globulin and 375-rad total-body irradiation. They observed successful engraftment, indicating the adequacy of the immunosuppressive regimen and apparent absence of graft-versus-host disease, but one patient died of infection and the other of an early return of leukemia.

**Technic of Marrow Transplantation**

In the laboratory, marrow has been administered by a variety of routes, including intravenous, intra-arterial, intraperitoneal and intramedullary. Van Bekkum studied the problem of the route of administration in the mouse and found the intravenous route to be the best since the intraperitoneal route required about 70 times as much marrow for successful engraftment. Recent studies of this problem in the monkey showed that two to three times as much marrow is required when it is given intraperitoneally as compared to intravenously. Although some patients with immunologic deficiency disorders have had successful engraftment after intraperitoneal administration, most investigators now use the intravenous route.

Histocompatible donors are most likely to be found among members of a patient's family, and, thus, living volunteers are at present the principal source of donors for marrow grafts. One unit of blood is obtained from the marrow donor a few days before the procedure, stored in acid citrate dextrose, and returned to the donor during the aspiration, thus avoiding exposure of the normal donor to the risks of blood transfusion from another person. Marrow is obtained by multiple aspirations from the iliac bones in an operating room under sterile conditions. Spinal anesthesia is preferred, but occasionally general anesthesia is indicated. When the needle point is inserted into the marrow cavity, vigorous suction is applied while the needle is rotated. The volume aspirated from each site is limited to 1 to 3 ml to minimize dilution with peripheral blood. Marrow is aspirated and transferred quickly into a beaker containing tissue-culture medium and heparin without preservative. The pooled aspirated marrow, usually 400 to 800 ml, is passed successively through stainless-steel screens of 0.3-mm and then 0.2-mm opening, screening produces a single-cell suspension permitting accurate cell counts and, most importantly, avoids potentially lethal marrow emboli. Cooling the marrow suspension is unnecessary and may lead to clumping of fat. The recipient must be monitored carefully during the intravenous infusion. Signs of fluid overload or strain on the right side of the heart dictate slowing of the infusion or, occasionally, phlebotomy before the infusion is completed. We have carried out marrow aspirations on more than 200 donors. Invariably, the procedure has been well tolerated. No long lasting effects are to be expected from the removal of a small fraction of a rapidly replicating tissue. The risk of anesthesia remains a legitimate concern.

**Supportive Care**

Before grafting, almost all marrow-graft recipients go through a period of no marrow function owing to their disease or to preparation for engraftment. After grafting, there is a period of 10 to 20 days before the graft begins to function. Effective supportive measures during these periods of marrow aplasia are absolutely essential to success in a marrow-transplantation program.

Before transplantation it is very important that blood products from family members should not be administered to potential recipients of sibling marrow transplants because of possible sensitization against non-HLA tissue antigens of the donor. An exception is that children of the prospective recipient may be used when the donor is to be a sibling of the recipient. After the marrow graft the family may be used as blood-product donors. The intensive use of one or two donors greatly reduces the potential exposure to cytomegalovirus, toxoplasma and hepatitis. All blood products infused into recipients of allogeneic marrow transplants are exposed to 1500 R of Co irradiation to prevent the proliferation of lymphoid cells, which might produce or enhance graft-versus-host disease.

Our policy is to attempt to maintain a circulating platelet count of greater than 20,000 per cubic millimeter. Platelet consumption due to infection or the underlying disease is a frequent problem. Platelets from HLA-matched siblings are probably not subject to immunologic destruction.

Available technics do not provide the same transfusion support capability for granulocytes as for platelets. Even small elevations of circulating granulocyte levels require very large numbers of infused cells owing to their dilution in the marginal pool, the short half-life (six hours) of normal granulocytes and their rapid utilization in the face of infection. Most groups involved in granulocyte transfusion therapy of infected agranulocytic patients have concluded that such transfusions are beneficial, but objective data supporting these conclusions are understandably scarce. The largest controlled studies were conducted by Gray and McCredie and their associates. If recipient sensitization to allogeneic granulocytes is suspected, the suitability of a donor can be assessed by determination of the survival of the potential donor's platelets. For our patients the granulocyte donor has usually been the marrow donor or another family member. Such donors are well motivated to tolerate the daily inconvenience. We initiate granulocyte transfusion therapy when a serious bacterial or fungal infection develops in an agranulocytic transplant recipient. Once started, the transfusions are continued daily until the recipient is able to support levels of circulating granulocytes higher than can be achieved by granulocyte transfusion. We are investigating the potential benefits of prophylactic granulocyte transfusion for uninfected agranulocytic patients in a controlled study.

We harvest granulocytes with two National Cancer Institute-IBM continuous-flow cell separators using arteriovenous shunts in the donor. This procedure is well tolerated by the donor and permits the processing of
larger quantities of blood on a daily basis. Approximately 18 ± 8.7 (± S.D.) × 10⁶ granulocytes can be collected daily by this technic. The granulocyte-rich buffy coat often contains sufficient platelets to satisfy the platelet requirements of the recipient. Reversible leukoadehesion with use of Leukopaks was developed by Djerassi.⁵⁸ Equipment for this procedure is commercially available and relatively inexpensive. There are reports of severe recipient reactions to Leukopak-collected granulocytes,⁵⁹ but this has not been a problem in our experience.

Because of the susceptibility to infection, it seems reasonable to isolate the patient from possible contact with pathogenic micro-organisms. Recent developments in techniques of laminar-air-flow isolation and of suppression of the gastrointestinal flora with nonabsorbable antibiotics have increased the ability to achieve the "sterile" patient.⁶¹,⁶² The observation that the clinical manifestations of graft-versus-host disease can be less severe in gnotobiotic mice than in normal mice has prompted the speculation that attempts to render human recipients of marrow transplants bacteria-free might reduce the frequency and severity of this complication. These procedures are complicated and expensive and involve patient discomfort and inconvenience. It is therefore very important that their benefits be accurately evaluated. Controlled studies of this problem are under way in our center, but no firm conclusions are available at present. As a practical matter, almost all our marrow transplants have been carried out with simple mask reverse isolation. In the absence of controlled studies, the published suggestion that marrow grafts should only be done in a sterile environment should be regarded as speculative.⁶³,⁶⁴

**Clinical Results of Marrow Transplantation**

**Immunodeficiency**

This review is concerned primarily with marrow transplantation in persons who have achieved immunologic maturity. Studies of marrow and thymus transplantation in patients with immunologic deficiency have provided important basic information, despite the rarity of these disorders. Details of these studies are provided in review articles.⁶¹,⁶² In reading these reviews, one must keep in mind that patients with severe combined immunologic deficiency are different from the cases in this report in two important respects: the disease represents an experiment of nature in which the recipient does not require immunosuppression to accept a graft; and because some myeloid marrow function is usually present, rapid marrow engraftment is not mandatory, permitting the use of very small numbers of marrow cells, given repeatedly if necessary. These differences permit the design of highly informative experiments that are not possible in patients with aplastic anemia or leukemia.

**Aplastic Anemia**

*Syngeneic (monozygotic twin) transplants.* Despite introduction of broad-spectrum antibiotics, androgens, corticosteroids, and support with cellular blood elements, the mortality in severe cases of aplastic anemia is 80 to 90 per cent, with many patients dying within the first three months of diagnosis.⁶⁶,⁶⁷ Treatment of this illness by infusion of marrow cells from a normal identical twin is of great interest since the fate of the infused hematopoietic cells might provide valuable information about the cause and the pathogenesis of aplastic anemia. Up to the present, 10 cases have been described involving patients with acquired aplastic anemia whose identical twins donated marrow.⁶⁸ Two patients died within a few hours or days of marrow infusion. In five of the remaining eight, signs of beginning marrow regeneration were observed one to two weeks after grafting, and full recovery followed. The three patients treated in Seattle are alive and well with normal hemopoietic function six to 12 years later.⁶⁹ Although proof of engraftment cannot be obtained in the syngeneic situation, these observations suggest that most cases of aplastic anemia are due to a persistent abnormality induced in the stem-cell population. Otherwise, infusion of normal, syngeneic stem cells would not be expected to correct the abnormality. The patient with marrow failure who is fortunate enough to have a normal identical twin is a natural candidate for marrow transplantation and should be treated promptly since this form of therapy is essentially devoid of risk and has a high probability of success.

*Allogeneic transplants.* Through the end of February, 1974, we had treated 37 patients with aplastic anemia with transplants of marrow from major-histocompatibility-complex-matched siblings.⁶⁸,⁷⁰ Briefly, the patients' ages ranged from three to 67 years, with a median of 19. Twenty-three of the 37 had aplastic anemia of unknown cause; in seven the disease was associated with drugs, and in four with hepatitis; in two it occurred after a prolonged period of acquired paroxysmal nocturnal hemoglobinuria and in one it was associated with Fanconi's syndrome. The duration of the anemia ranged from one to 53 months, with a median of three months. Thirty-one patients had received therapy with androgenic steroids and had failed to respond. All but two had received multiple transfusions from unrelated donors before transplantation. Eight patients had received transfusions from parents or siblings. Eighteen patients were refractory to random platelet transfusions. Seventeen were infected at the time of admission. Granulocyte levels at admission ranged from 0 to 1000 and platelet counts from 1000 to 22,000 per cubic millimeter. Thirty-six patients were estimated to have less than 10 per cent normal megakaryocyte numbers in the marrow, 30 had less than 10 per cent granulocyte precursors, and 29 had less than 10 per cent erythroblast precursors at the time of admission.

Twenty-eight patients were conditioned for marrow grafting by administration of cyclophosphamide, 50 mg per kilogram, intravenously on each of four successive days. Nine patients were conditioned by a 1,000-rad midline tissue exposure of total-body irradiation. The day of marrow infusion was designated "day 0." After marrow grafting, methotrexate was administered intermittently as described below. Survival curves for these patients are shown in Figure 1.

Three patients died too early to evaluate success or failure of marrow grafting; one died on the day of grafting of congestive heart failure, possibly related to cyclophos-
phamide cardiac toxicity; two died on the sixth and eighth days from bacterial infection. One patient had no evidence of marrow engraftment and died on the 24th day of infection. The marrow at autopsy was extremely hypocellular.

Thirty-three patients gave evidence of marrow engraftment as indicated by rising peripheral blood counts between the 10th and 21st days after marrow infusion. Examination of marrow aspirates and biopsies confirmed marrow engraftment. Twenty-one of the 33 had donors of opposite sex. Cytogenetic analyses of marrow and peripheral blood cells carried out as early as the 14th day after grafting proved engraftment. Only cells of donor karyotypes have been found up to the present in surviving patients. In other patients, other blood genetic markers such as red-cell antigens and enzymes were used to monitor allogeneic engraftment. After initial marrow engraftment, six patients rejected their grafts, with progressively falling peripheral blood counts leading to marrow hypoplasia. Five of the six died between the 33rd and 67th days with infection. One of the six showed autochthonous marrow recovery as evidenced by cytogenetic analyses of marrow and peripheral blood cells and is well more than one year later. Of the patients with a graft, one died with a fungal infection on the 18th day, three died from cytomegalovirus pneumonia between the 54th and 91st days and one died of unknown causes on the 427th day. Six patients died with graft-versus-host disease between the 19th and 95th days. Sixteen patients are alive, with functioning grafts.

The results show that normal allogeneic hematopoietic stem cells can repopulate the marrow in patients with aplastic anemia and that the disease is usually related to a failure of the hematopoietic stem cells. An exception to this rule may be the one patient with autochthonous marrow recovery after rejection of the marrow transplant from his sister. Our experience shows that long-term stable chimerism is possible in man. Clearly, major problems remain to be solved. Even so, the survival and hematologic reconstitution of almost half our patients with advanced aplastic anemia treated by allogeneic marrow grafts compare favorably with results obtained by conventional management of this disease. Initially, we restricted this procedure to critically ill patients. The results, however, indicate that marrow transplantation has emerged as a definitive form of therapy for aplastic anemia provided an HL-A matched sibling can be identified as a potential marrow donor. Marrow grafting should be considered before major infection and refractoriness to blood transfusions occur. Other centers have now reported successful marrow grafts in aplastic anemia that confirm this suggestion. 102, 103

Acute Leukemia

Syngeneic (monozygotic twin) transplants. Since lethal total-body irradiation can exert a definite antileukemic effect, and since syngeneic marrow infusion can prevent death from the irradiation-induced aplasia, the rare leukemic patient with advanced disease who is fortunate enough to have a normal identical twin is the logical candidate for attempts to eradicate the leukemia by total-body irradiation and twin marrow transplantation. Very early encouraging results in mice prompted Thomas et al. 104 to try such an approach in three patients with acute lymphoblastic leukemia. Although normal hematopoiesis was restored, leukemia recurred within seven to 12 weeks. A fourth patient with acute lymphoblastic leukemia received cytosine arabinoside before the total-body irradiation and methotrexate after the marrow transplant, but still relapsed within seven weeks (Table 1).

Beginning in 1969 potential "immunotherapy" was added to the basic regimen of total-body irradiation and twin marrow with the hope of delaying leukemic recurrence. 105 It was based on principles outlined in the above section on studies in rodents and on studies supporting the existence of human leukemia-associated antigens. Since we would not expose normal twins to tumor material, the immunotherapy after total-body irradiation and marrow transplantation consisted of infusion of normal-twin buffy-coat lymphocytes three times a week for three weeks and weekly subcutaneous injections of lethally irradiated, autologous, leukemic cells, in the hope of providing a continual antigenic stimulus to the infused donor lymphocytes. The leukemic cells were stored at -180°C in 10 percent dimethyl sulfoxide and irradiated with 10,000 rad to prevent cell replication. The results obtained in three patients treated with total-body irradiation, marrow transplantation and immunotherapy are

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<th>Therapy</th>
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</tr>
<tr>
<td>Cyclophosphamide +</td>
<td>7</td>
<td>5</td>
<td>&gt; 3, 4, 4.1, 6.1</td>
<td>2</td>
</tr>
<tr>
<td>Irradiation +</td>
<td></td>
<td></td>
<td>&gt; 18</td>
<td>2</td>
</tr>
<tr>
<td>Cyclophosphamide +</td>
<td>13*</td>
<td>10</td>
<td>3, 4, 5, 7</td>
<td>6</td>
</tr>
<tr>
<td>Irradiation +</td>
<td></td>
<td></td>
<td>&gt; 8, &gt; 16</td>
<td></td>
</tr>
<tr>
<td>Immunotherapy</td>
<td></td>
<td></td>
<td>&gt; 21, &gt; 31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt; 32, &gt; 49</td>
<td></td>
</tr>
</tbody>
</table>

*Death at 4 mo of pneumonitis in remission.

1 Patient died of pneumonitis at 2 mo in remission.
shown in Table 1. A 15-year-old patient with acute lymphoblastic leukemia relapsed within a month, a 26-year-old patient with acute myelogenous leukemia went into remission but died of interstitial pneumonitis at seven weeks, and a 33-year-old patient with acute myelogenous leukemia relapsed at 10 months.

We assumed that the addition of a high dose of chemotherapy to the treatment regimen would be beneficial by decreasing the tumor load to be handled by the total-body irradiation or immunotherapy or both. Therefore, 29 subsequent patients received cyclophosphamide (60 mg per kilogram on each of two days), total-body irradiation (1000 rad), twin marrow transplants and, in 13 cases, immunotherapy. All patients had received chemotherapy and were considered to be in the terminal phase of the illness. The seven patients who did not receive immunotherapy either had not had readily accessible tumor cells for storage or had normal-twin donors who were unable to donate regularly the necessary buffy-coat cells. Thus, this was not a randomized study to determine the contribution, if any, of immunotherapy to the overall results. The results are shown in Table 1. One patient died of hepatitis too early to be evaluated, and two failed to clear their marrow of leukemic cells. Seventeen experienced complete remission. Eight of them (three with acute lymphoblastic, four with acute myelogenous, and one with lymphosarcoma leukemia) remain in complete remission at three to 49 months without any maintenance chemotherapy. All the normal-twin donors remain in excellent health without evidence of hematologic neoplasia.

As predicted from rodent models, although normal hematopoiesis was restored in our twin transplant recipients, the principal problem was that of resistance of the hematologic neoplasms in almost half our patients, as reflected by persistence of leukemic cells or recurrence of leukemia after several months of remission. This resistance has also been a problem in four patients with leukemia treated by others with high-dose combination chemotherapy with marrow infusion from a normal twin—all relapsed within a few months.\textsuperscript{39,109} We are now adding intensive combination chemotherapy before the cyclophosphamide and total-body irradiation to further reduce the tumor burden. Our results are sufficiently encouraging to recommend this approach to any patient who has an identical twin and hematologic neoplasia even before the patient becomes demonstrably resistant to all chemotherapy. Furthermore, this approach may also be applicable to the treatment of other tumors such as lymphomas, neuroblastomas, and anaplastic carcinomas known to be sensitive to high doses of chemotherapy and total-body irradiation.

\textbf{Allogeneic transplants:} The majority of marrow grafts for acute leukemia have been performed between ABO-compatible, HLA-identical siblings and siblings not reactive in mixed leukocyte culture. The main differences in the approach to marrow grafting have centered on methods of conditioning the patient for the marrow graft and of post-grafting immunosuppression.\textsuperscript{14} Table 2 details the approaches described in reports published by others since 1970. Of the conditioning regimens so far tried, reproducible, high-level chimerism and sustained remission have been obtained only with cyclophosphamide in doses of at least 45 mg per kilogram per day for four days, high-dose chemotherapy (BACT)\textsuperscript{19} and, as will be described, total body irradiation alone or in combination with cyclophosphamide. Conditioning with antilympho-

### Table 2. Summary of Recently Reported Results of Marrow Transplantation for Acute Leukemia Using Irradiation, Cyclophosphamide, Anti-Lymphocyte Serum, or Combination Chemotherapy for Conditioning.\textsuperscript{*}

<table>
<thead>
<tr>
<th>Conditioning Regimen\textsuperscript{*}</th>
<th>No. of Patients</th>
<th>Gravy &quot;Takes&quot;</th>
<th>Post-Transplant Immunosuppression</th>
<th>No. Surviving in Remission</th>
<th>Survival (Days)</th>
<th>Cause of Failure</th>
<th>Source of Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY I</td>
<td>1\textsuperscript{1}</td>
<td>0</td>
<td>None</td>
<td>0</td>
<td>90</td>
<td>Leukemia</td>
<td>Meuwissen et al\textsuperscript{107}</td>
</tr>
<tr>
<td>CY II</td>
<td>8\textsuperscript{1}</td>
<td>7</td>
<td>CY\textsuperscript{(8)}</td>
<td>0</td>
<td>13-215</td>
<td>GVHD (6), infection (3), leukemia (1)</td>
<td>Santos et al\textsuperscript{26}</td>
</tr>
<tr>
<td>CY II</td>
<td>1\textsuperscript{1}</td>
<td>1</td>
<td>None</td>
<td>77</td>
<td></td>
<td>Infection</td>
<td>Pruzanski et al\textsuperscript{24}</td>
</tr>
<tr>
<td>CY III</td>
<td>8\textsuperscript{1}</td>
<td>7</td>
<td>CY (1) or MTX (6)</td>
<td>10-602</td>
<td></td>
<td>GVHD (1), infection (1) leukemia (6)</td>
<td>Graw et al\textsuperscript{27}</td>
</tr>
<tr>
<td>950 rad</td>
<td>1\textsuperscript{1}</td>
<td>1</td>
<td>MTX</td>
<td>33</td>
<td>60-180</td>
<td>GVHD</td>
<td>Graw et al\textsuperscript{27}</td>
</tr>
<tr>
<td>ALS</td>
<td>10\textsuperscript{1}</td>
<td>5</td>
<td>None</td>
<td></td>
<td></td>
<td>Partial chimerism, no antileukemic effect</td>
<td>Amiel et al\textsuperscript{23}</td>
</tr>
<tr>
<td>ATG + 375 rad</td>
<td>2\textsuperscript{1}</td>
<td>2</td>
<td>MTX</td>
<td>54-122</td>
<td></td>
<td>Leukemia (1), infection (1)</td>
<td>Gengenbier et al\textsuperscript{109}</td>
</tr>
<tr>
<td>BACT</td>
<td>4\textsuperscript{1}</td>
<td>3</td>
<td>CY (3), MTX (1)</td>
<td>1</td>
<td>3-6-600</td>
<td>GVHD (1), infection (1)</td>
<td>Graw et al\textsuperscript{29}</td>
</tr>
</tbody>
</table>

\textsuperscript{*} Figures in parentheses represent no. of patients. CY denotes cyclophosphamide, ALS antilymphocyte serum, ATG antilymphocyte globulin, MTX methotrexate, BNCU 1, 3-biot-
\textsuperscript{(2-chloroethyl)-1-aminomethane, & GVHD graft-vs-host disease.}

\textsuperscript{1} CY: Donor antigen (IU whole blood) on day -2; CY 37.5 mg/kg on days -2 & -1.

\textsuperscript{1} CY II: Donor antigen (IU whole blood) on day -5; CY 30-60 mg/kg on days -4, -3, -2, & -1.

\textsuperscript{1} CY III: Donor antigen on day -5; CY 45 mg/kg on days -4, -3, -2, & -1.

\textsuperscript{1} ALS: 20-30 ml of bone marrow infused on days 1-3.

\textsuperscript{1} BACT: CY 45 mg/kg on days -4 to -1, cytosine arabinoside & thioguanine each at 100 mg/m\textsuperscript{2} on day -1, 7 doses; BNCU 200 mg/m\textsuperscript{2} on day -1.

\textsuperscript{1} HLA-identical siblings.

\textsuperscript{1} CY: 5-7.5 mg/kg on days 6, 8, 10, 12 & 14 post-transplant 2 oral CY for 3mo.

\textsuperscript{1} MTX: 10 mg/m\textsuperscript{2} on days 1, 3, 6, 11 & then every week.

\textsuperscript{1} GVHD were not HLA-identical.
cyte serum alone has not demonstrated an antileukemic effect or high-level chimerism.

In Seattle, 1000-rad total-body irradiation, initially alone and subsequently with the addition of cyclophosphamide and, more recently, cytoreduction as described, have been used as the conditioning regimen in the 70 patients receiving marrow grafts for acute lymphoblastic or acute myelogenous leukemia between July 1969, and June 1974 (Table 3). A review of this five-year experience illustrates the changing problems but the definite progress evident in marrow grafting for acute leukemia.

Table 3. Allogeneic Marrow Transplants for Acute Lymphoblastic (ALL) and Acute Myelogenous (AML) Leukemia Using HL-A Matched Sibling Donors (Seattle Experience).

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Patients</th>
<th>Remission</th>
<th>Recurrent Leukemia</th>
<th>Survival of Patients Now in Remission (Mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt; 3 Mo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>34</td>
<td>19</td>
<td>11*</td>
<td>49, 31, 26, 19, 13, 6, 3, 4, 3</td>
</tr>
<tr>
<td>AML</td>
<td>36</td>
<td>14</td>
<td>4</td>
<td>29, 23, 16, 12, 7, 3, 4, 3, 3, 3</td>
</tr>
<tr>
<td>CML-BC</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Of these patients were prepared with total-body irradiation only.
†Patient relapsed with central-nervous-system leukemia at 18 mo; all other patients relapsed by 3 mo.
‡Chronic myelogenous leukemia in blast crisis.

Thirty-six patients, two to 56 years of age (average of 23 years) received marrow grafts for acute myelogenous leukemia, and 54 patients, five to 22 years of age (average of 11 years) for acute lymphoblastic leukemia. Relevant to evaluating this approach is the clinical condition of these patients at the time of grafting. All patients received transplants at a time when conventional and experimental chemotherapy was failing. The duration of disease from diagnosis averaged 24 months in patients with acute lymphoblastic leukemia, spanning an average of three relapses, and in acute myelogenous leukemia, it averaged 10 months, with 15 patients not achieving a remission with chemotherapy. Overall, 64 of the 70 patients were in relapse, 68 had previously been transfused with red cells or platelets (or both), and 22 were infected at the time of transplantation.

Two patients died in less than 10 days, and five did not show engraftment. Sixty-three obtained functional grafts demonstrated by rapidly rising peripheral counts, histologic evidence of a graft, and, when possible, cytogenetic analysis and red-cell or white-cell antigen or enzyme studies. In 46 of these 63 patients (73 per cent) graft-versus-host disease developed, ranging from an erythematous skin rash lasting only several days to the fatal syndrome of a desquamating erythematous skin rash, gross liver-function abnormalities and profuse diarrhea. There was no difference in the incidence of graft-versus-host disease in the two disorders — 21 of 31 patients with acute lymphoblastic and 25 of 32 patients with acute myelogenous leukemia.

The overall survival data for this group of patients are shown in Figure 2. Although the median survival for patients with the two types of leukemia remains short, the five-year period is characterized by marked improvement in supportive care of patients and more effective conditioning regimens. Analysis of the results according to the year of transplantation demonstrates the progress now evident in two respects: the prolongation of survival beyond the first 50 days; and the increasing number of long-term survivors. Thus, the percentage of patients surviving beyond 50 days has increased from less than 50 per cent in those receiving transplants between 1969 and 1971 to 100 per cent in those with transplants in the first six months of 1974.

Relapse was the basic cause of failure in 15 patients, four with acute myelogenous and 11 with acute lymphoblastic leukemia. Included are four patients (two in each group) whose leukemic cell population was so refractory that serial marrow aspirations demonstrated persistence of blast cells. Such refractoriness suggests a spectrum of degrees of reduction of the leukemic cell load. One could anticipate, therefore, that a substantial fraction of the patients might retain a malignant cell population too large for a putative antileukemic effect of a marrow graft to do much good. In fact, we have observed four patients in whom leukemia recurred in seven to 23 weeks after marrow engraftment, and in whom cytogenetic evidence showed that the recurrence was composed of cells derived from the original host leukemia. Furthermore, the more recent aggressive conditioning regimens appear to have modified the relapse rate. Of the first six patients with acute lymphoblastic leukemia prepared with total-body irradiation alone, five had evidence of recurrence (including a child who died primarily of bilateral interstitial pneumonia and had extramedullary microscopic evidence of leukemia). With the subsequent addition of high-dose chemotherapy to the conditioning regimen, only six of 29 patients with acute lymphoblastic leukemia have shown a recurrence. As the median survival increases, however, more cases of recurrence may also become evident, although relapse beyond seven months has occurred in only one patient.

Nineteen of the 70 patients remain in complete remission beyond three months, nine for one to four years after transplantation. From the data presented, indications are that such a remission rate is increasing (Fig. 2). In many patients with acute myelogenous leukemia and adult acute lymphoblastic leukemia, and after relapse in acute leukemia of any kind, long-term unmaintained remissions are not possible with available chemotherapy. The results with marrow transplantation, far from ideal, are nonetheless encouraging and offer a therapeutic opportunity for the patient with a suitable donor whose leukemia by virtue of type or relapse augurs a predictably short prognosis.

The results of marrow grafting for the blastic crisis of
chronic myelogenous leukemia cannot yet be evaluated. Two patients died early of infection, and one with a graft is in remission after four months (Table 5).

Current Results in Miscellaneous Conditions

Three other applications of marrow grafting have long been recognized: radiation accidents; solid malignant tumors that are particularly sensitive to chemotherapy and radiotherapy; and genetic disorders such as thalassemia and sickle-cell disease.

Apart from transplants after radiation accidents, there are to date no published reports of long-term survival after allogeneic transplants for the disease categories listed. In these disorders, either the transplantations have been performed before the recognition of the importance of HLA and mixed-leukocyte-culture matching, patients having succumbed to overwhelming infections in the early post-grafting period, or there has been no evidence of engraftment.

Within our center allogeneic marrow grafting has been carried out in single patients with neuroblastoma, malignant histiocytosis, and Hodgkin's disease. All succumbed within 40 days of transplantation, two from overwhelming sepsis and one from viral pneumonia.

A major factor limiting the total eradication of a disseminated malignant neoplasm by current available chemotherapy and radiotherapy is irreversible damage to the marrow. Transplantation of marrow should remove this barrier and would permit the administration of much larger doses of these agents. Neuroblastoma, lymphosarcoma, Hodgkin's disease and oat-cell carcinoma are examples of tumors that appear to be particularly sensitive to chemotherapy and to radiotherapy, and in such neoplasms the application of marrow transplantation may enable potentially curable doses to be given.

Two approaches are feasible: the infusion of the patient's own normal marrow aspirated and stored before chemotherapy and radiotherapy (autologous marrow infusion); or the use of marrow from a major histocompatibility-complex-matched donor. In the late 1950's there were several case reports of remissions with use of autologous marrow infusion after high doses of chemotherapy or radiotherapy or both. Death of most patients from bacterial sepsis and the doubt whether marrow recovery in the long-term survivors was in any way related to the marrow infusion led to a general loss of interest in this technic. The development of methods for long-term storage of marrow at -180°C in dimethyl sulfoxide, better chemotherapeutic agents, and more effective supportive care of patients have renewed interest in this form of therapy, which, like identical twin transplants, does not carry the risk of rejection or graft-versus-host disease. Currently, we are storing marrow from patients with chronic myelogenous leukemia early in their disease. When blast crisis occurs, the patient will be treated with intensive chemotherapy and radiotherapy followed by return of the stored marrow, with the intent of restoring the chronic disease. Preliminary results indicate feasibility.

Current Problems

Success or Failure of Marrow Engraftment and Marrow-Grat Rejection

A review of the literature of human marrow transplantation before 1967 showed a high incidence of complete failure of engraftment. Failure of initial allogeneic engraftment is no longer a major problem. Excluding five patients who died too soon to be evaluated, 33 of 34 grafts
in patients with aplastic anemia were successfully established, and 63 of 68 in acute leukemia were successful. The quantity of marrow infused ranged from 1.1 to 10.9 x 10^6 per kilogram of recipient body weight (Table 4). Despite this range of one order of magnitude, there was no correlation between marrow dose and success or failure of engraftment nor of nadir of white-cell count or time to recovery above 1000 per cubic millimeter. Although there has been concern about the dose of marrow cells required for engraftment, precise determination of this number seems to be neither practical nor necessary.

Experiments in canine literates matched at the major histocompatibility complex have shown that prior exposure to transfusion of whole blood from the marrow donor may jeopardize the success of a subsequent marrow graft even in this "compatible" donor-recipient combination. Data similar to those in dogs have been reported in irradiated mice and also in mice treated with cyclophosphamide. Presumably, rejection was due to immunization of the recipient to histocompatibility antigens on platelets and leukocytes. With blood transfusions from random donors or from family members other than the marrow donor, immunization of the recipient and rejection of the graft might be expected only when the marrow donor and the transfusion donors share "minor" histocompatibility antigens not present in the recipient. The results of allogeneic marrow grafting for the treatment of aplastic anemia have shown that patients given transplants from family members have a high rate of failure of marrow engraftment or of marrow-graft rejection. The two patients who had not been transfused before grafting had prompt engraftment and are among the long-term survivors. One patient who rejected his graft had had transfusions from both parents, which should have exposed him to the risk of sensitization to all family "minor" transplantation antigens that he did not inherit. Although Mathé et al. had earlier reported an apparent harmful effect of transfusions before grafting in patients with leukemia, we have not observed graft rejection in our patients with acute leukemia, and most of the failures of engraftment occurred in the earlier patients. More aggressive modern chemotherapy is one explanation for this difference as well as the difference between acute leukemia and aplastic anemia.

At present no reliable laboratory tests are available to determine which patient is sensitized against his HL-A-

matched marrow donor although research on this problem is in progress. Recent data using DLA-incompatible, unrelated donor recipient pairs of dogs indicate that the presensitized state can be abrogated by a combination of procarbazine and rabbit antidiog antithymocyte serum before total-body irradiation resulting in successful marrow engraftment in most cases. We have initiated a randomized study in patients with aplastic anemia to determine the role of pre-treatment with procarbazine and antithymocyte globulin. Initial results have shown that the regimen is well tolerated and that successful engraftment is possible.

**Graft-versus-Host Disease**

The distinction between illness due to the active immunologic assault of donor lymphoid cells against host target organs and the consequences of this assault, deranged organ function and infection, is indeed subtle and, in this discussion, both are considered a part of graft-versus-host disease. For unknown reasons, the principal target organs of graft-versus-host disease in both animals and man are skin, gastrointestinal tract and liver. Despite the use of sibling donors matched at the major histocompatibility complex and despite post-grafting immunosuppression, graft-versus-host disease has occurred in approximately 70% of patients with successful marrow grafts. Evidently, compatibility or incompatibility for histocompatibility regions other than the major histocompatibility complex are important determinants of the graft-versus-host disease. There have been too few successful marrow transplantations between siblings not matched at the major histocompatibility complex to know the frequency of graft-versus-host disease in this situation, but, from studies in animals as well as from the results of marrow grafting in immunodeficient children, it seems reasonable to expect that graft-versus-host disease (and graft rejection) will be major problems. We described a marrow transplant involving a one-antigen mismatch. Graft-versus-host disease was severe but not more severe than that seen in some transplants matched at the major histocompatibility complex.

**Clinical graft-versus-host disease.** The proposed clinical staging of the disease is shown in Table 5. The initial organ

| Table 4. Marrow Cells Infused and Days Required for White-CellCount (WBC) to Go above 1000 per Cubic Millimeter. |
|-----------------|----------------|----------------|----------------|
| **DISEASE**     | **No. ANALYZED** | **Marrow Cells Infused** | **Day WBC > 1000** |
|                 | **MEDIAN** | **RANGE** | **MEDIAN** | **RANGE** |
| Aplastic anemia | 34        | 2.6      | 1.1-9.4  | 17        | 5-37     |
| Acute lymphoblastic leukemia | 31 | 3.3  | 1.4-10.9 | 21 | 11-35 |
| Acute myelogenous leukemia | 32 | 3.0  | 1.1-6.4  | 22 | 12-37 |

| Table 5. Proposed Clinical Stage of Graft-versus-Host Disease According to Organ System. |
|-----------------|----------------|----------------|----------------|
| **STAGE**       | **SKIN**       | **LIVER**      | **INTESTINAL TRACT** |
| Maculopapular rash <25% of body surface | Bilirubin 2-3 mg/100 ml | > 500 ml diarrheaday |
| Maculopapular rash 25-30% body surface | Bilirubin 3-6 mg/100 ml | > 1000 ml diarrheaday |
| Generalized erythroderma | Bilirubin 6-15 mg/100 ml | > 1500 ml diarrheaday |
| Generalized erythroderma with bullous formation & desquamation | Bilirubin > 15 mg/100 ml | Severe abdominal pain, with or without ileus |
involved in almost all cases is the skin. Biopsy is mandatory for diagnosis since not all rashes in these patients are due to graft-versus-host disease. Hepatic and intestinal involvement usually appears several days after the rash. Intestinal involvement is mainly in the form of diarrhea but may progress to abdominal pain and ileus. Liver disease is manifested by rises in bilirubin (mainly conjugated). Serum glutamic oxalacetic transaminase is usually in the range of 150 to 750 IU. Rises in alkaline phosphatase are also observed especially in chronic graft-versus-host disease. Intestinal and hepatic abnormalities are not unique to this situation. Therefore, alterations in function of these organs should be attributed to graft-versus-host disease only when its involvement of the skin is clearly established or when there is biopsy confirmation in gut or liver. Fever, wasting and a decreased performance status are also regularly seen with severe involvement. Although liver may be a manifestation of graft-versus-host disease per se, it is most commonly due to an associated infection, often an occult viral infection.

We and others have attempted to classify the overall severity of clinical graft-versus-host disease on a grading system ranging from 0 to IV (Table 6). Currently, analysis of survival among our patients suggests that patients can be grouped in two ways: those without clinically evident graft-versus-host disease (Grade 0) and those with graft-versus-host disease involving skin (Grade I) show a survival of 55 percent, whereas patients with Grades II to IV have a survival of only 15 percent. Its strong association with infection indicates that graft-versus-host disease itself is a critical factor contributing to the poor immunologic reactivity, debility and compromised mucosal barriers in marrow-graft recipients. The grading system may become more meaningful as advances are made in the treatment of graft-versus-host disease and accompanying infections.

Chronic graft-versus-host disease characterized by a more indolent clinical course, with involvement of skin, liver and intestinal tract, has been observed in three long-term survivors. This picture may appear after complete or partial resolution of acute graft-versus-host disease. Whether the chronic form is the result of an immunologic attack of donor lymphoid cells against host tissue or of deficient immunologic reactivity of the graft, with accompanying opportunistic infections, or of a combination of both is not well understood at present.

Table 6. Overall Clinical Grading of Severity of Graft-versus-Host Disease.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Degree of Organ Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+ to ++ skin rash; no gut involvement; no liver involvement; no decrease in clinical performance.</td>
</tr>
<tr>
<td>II</td>
<td>+ to +++ skin rash; + gut involvement or + liver involvement (or both); mild decrease in clinical performance.</td>
</tr>
<tr>
<td>III</td>
<td>++ to +++ skin rash; ++ to +++ gut involvement or ++ to +++ liver involvement (or both); marked decrease in clinical performance.</td>
</tr>
<tr>
<td>IV</td>
<td>Similar to grade III with + to +++ organ involvement &amp; extreme decrease in clinical performance.</td>
</tr>
</tbody>
</table>

Histologic criteria. Table 7 summarizes the proposed histopathologic staging of graft-versus-host disease based on scale ranging from + to + + +. Hepatic involvement was characterized by degeneration and eosinophilic necrosis of parenchymal cells and degeneration and necrosis of the epithelium of small bile ducts. Because the effect on parenchymal cells was variable whereas small bile ducts were consistently involved, the grading was correlated with the percentage of pathologically altered small bile ducts.

Serial biopsies of the skin are easily obtained, but biopsies of the gut and liver cannot be done regularly and the histopathologic stage may therefore require revision. Histopathologic staging varied between organs and did not always correlate with the clinical grade except for mild or severe graft-versus-host disease.

Prevention and treatment. From the animal studies as well as from the clinical marrow-grafting experience it has become clear that some form of immunosuppressive therapy must be used after grafting to diminish or prevent graft-versus-host disease. The literature on this topic has recently been reviewed. To be effective, treatment with immunosuppressive agents must be started before graft-versus-host disease has become apparent. Of the many agents studied, methotrexate and cyclophosphamide have been found to be useful. Methotrexate was found to ameliorate graft-versus-host disease in mice, dogs and monkeys when given immediately after grafting. Studies in dogs have shown that methotrexate was most effective when continued for a long time (three months), and stable grafted long-term chimera (measured in years) was achieved in some DLA incompatible recipients. Cyclophosphamide had some effect in mice, rats and monkeys, but was ineffective in dogs. Studies using cycsine arabinoside, procarba- zine, 6-mercaptopurine, and antilymphocyte serum in the immediate post-grafting period have been disappointing.
Prevention of graft-versus-host disease has been attempted with use of antilymphocyte serum given just before grafting in rodents, dogs, and monkeys, with only slight prolongation of survival.

Our current post-grafting regimen in human patients consists of methotrexate, 15 mg per square meter on the first day and 10 mg per square meter on the third, sixth, and 11th days and weekly thereafter for the first 100 days.96,108 Santos et al. have used cyclophosphamide, 7.5 mg per kilogram for five doses on alternate days, beginning on the first day after marrow grafting, followed by doses at irregular intervals.96 The frequency of fatal graft-versus-host disease in patients given a marrow graft from an HLA-matched sibling and no post-grafting immunosuppression is unknown. Despite post-grafting immunosuppression with either methotrexate or cyclophosphamide, severe and fatal graft-versus-host disease has been observed in 10 to 20 per cent of human marrow-graft recipients.

Surprisingly enough, only a few studies have been reported in which established graft-versus-host disease was treated either with cytostatic agents or with antilymphocyte serum. For the most part, these studies were negative—i.e., the agents used failed to influence the course of events or the authors failed to document sustained chimerism. A review of these studies has been presented recently.109 An exception was the study by Owens and Santos, who inoculated C57BL/6 × DBA 2 F1 hybrid mice with BALB/c spleen cells and were able to suppress clinically established graft-versus-host disease by cyclophosphamide.27 Cyclophosphamide was used to treat established graft-versus-host disease in a patient given a graft from an HLA-matched sibling and was ineffective.100

On the basis of encouraging studies in the canine model we have recently started using antithymocyte globulin for treatment of human patients with graft-versus-host disease.128 With this therapy, 12 of 19 patients showed complete resolution of the graft-versus-host disease, five showed improvement of most organ systems involved, and two showed no changes except for improvement in skin lesions. Six of the 19 became long-term survivors. Five of the six are alive now between 232 and 865 days after grafting, and one died on the 346th day with chronic respiratory failure. Of the remaining 13 patients, 11 died of interstitial pneumonitis of predominantly viral origin, and two died with fungal and bacterial infections. These findings demonstrated the partial effectiveness of antithymocyte globulin in reversing human graft-versus-host disease. This treatment of established graft-versus-host disease certainly does not represent the solution of the problem, but it appears to have modified the grim outlook in a hopeful way. It is obvious, however, that continued animal and clinical research efforts have to be directed to a better understanding of the nature and control of graft-versus-host disease.

Albumin-gradient cell-separation techniques have been used in an attempt to eliminate immunocompetent cells from the marrow inoculum while retaining the hematopoietic stem cell.28 Proof, however, that this approach is useful in modifying graft-versus-host disease in grafts between random bred non-rodenl animals is missing. The technic has been applied in a number of human transplants.91 The result was either failure of engraftment or death from graft-versus-host disease when histo-incompatible marrow was used. Aside from the technical problems, a number of theoretical reasons would argue against the efficacy of "stem-cell" separation technic. Even if it should be possible to separate completely lymphoid cells from stem cells, it is likely that the recipient still might be susceptible to the late form of graft-versus-host disease, which is presumably caused by subsequent regeneration of reactive lymphoid cells derived from the "common" stem cell. If there is not a "common" stem cell giving rise to lymphoid cells, successful separation would create a recipient who is severely deficient in immune function and thus might succumb to infection. At present, therefore, stem-cell separation does not appear to be a promising approach.

Infection After Engraftment

Leukopenia, immunoincompetence and graft-versus-host disease have, as their principal consequence, infection, which is the usual proximate cause of death. These complications may overlap in time and clinical manifestation, but it is possible to discern time periods in which one of the other underlying causes predominates.

The infectious hazards of agranulocytosis are maximally apparent during the two to three weeks immediately after transplantation. In a series of 71 allogeneic marrow-transplant recipients who survived for at least 50 days, and who achieved engraftment, the median number of days with a granulocyte count of < 100 per cubic millimeter was 11, and that of patients with a count of < 500 was 18. The pattern of infection in agranulocytic patients has been well delineated,121 mostly in patients with acute leukemia, and infectious-disease experience in marrow-transplant patients during the immediate post-grafting period conforms closely to this pattern. Bacterial and candidal infections are particularly frequent and serious in agranulocytic patients. Gram-negative bacteremia is a common event. There is convincing evidence that antibiotic therapy is less effective in curing infectious disease in patients lacking granulocytes and that the apparent complete resolution of an infectious process may be insecure unless adequate granulocytes are present. Organisms usually considered of low virulence acquire enhanced consequence in the absence of granulocytes. The frequency of systemic candidiasis is difficult to assess since techniques for its detection are of low sensitivity. Mucosal infections due to candida, however, are common. Bacterial infection appropriately treated rarely jeopardizes the achievement of successful engraftment, even in patients infected at the time the facilitation procedure is initiated, but it does cause considerable morbidity. Of 47 patients surveyed in a recent review,122 only two died from bacterial disease in the first 21 days, but in 22 bacteremia developed. Aside from the role of granulocyte transfusion the recently improved survival prospects of infected agranulocytic patients owe much to the development of new and more po-
tent antibiotics properly administered.

Because of decreased immune reactivity in these patients, it is not surprising that the incidence of infection, particularly viral disease, is increased during the first 100 days after transplantation. Perhaps the most troublesome single infectious complication is that of interstitial pneumonia. The syndrome has occurred at any time during the first four months after transplantation, but has its usual onset after the establishment of a functioning marrow graft. The overall incidence among 114 marrow-graft recipients in Seattle over the time period from 1969, through June, 1973, is 28 per cent. However, in the 24 identical-twin transplants in this series, the condition was documented in only three patients (13 per cent). Furthermore, as previously discussed, some allogeneic marrow recipients died without achieving effective hematopoietic function (or after marrow-graft rejection) in the period before the risk of interstitial pneumonia is very great. If these are excluded, and we consider only patients who achieved functional allogeneic transplants, the incidence of this syndrome is closer to 50 per cent. Mortality among this group of patients, death being primarily due to respiratory insufficiency from progressive pulmonary infiltration, is distressingly high at between 50 to 60 per cent. Whereas all groups of allogeneic marrow-graft recipients have contracted this syndrome, both the statistics on incidence and particularly those on mortality are worse in patients with grades II to IV graft-versus-host disease. In this connection, an apparent increase in incidence of interstitial pneumonia in marrow-transplant recipients has been reported in Seattle. However, it remains to be established whether this observation reflects a genuine increase in the attack rate or a manifestation of the enhanced chronicity of severe and moderate graft-versus-host disease (producing a longer period of maximum risk) resulting from the introduction of new methods of support and immunosuppressive treatment discussed above.

From the standpoint of cause about half the patients dying with interstitial pneumonia have unambiguous evidence of disseminated cytomegalovirus in the form of characteristic inclusion bodies on histologic examination of the lungs and other organs, as well as isolation of the organism in culture. A total of six histologically proved cases of Pneumocystis carinii has been noted at autopsy, three of which were found in the presence of cytomegalovirus. In two patients lethal varicella-zoster pneumonia developed. No recognizable pathogens have been associated with the remainder of the fatal interstitial pneumonias, although these are histologically indistinguishable from those caused by cytomegalovirus and pneumocystis (except for the specific signs of these agents). Of marrow-graft recipients who gave clinical evidence of interstitial pneumonia and subsequently recovered, about half also had serologic or cultural (or both) evidence of cytomegalovirus infection. The cause in the other half remains obscure.

A retrospective review of cytomegalovirus infection and serologic response among marrow-graft recipients provided the following observations: cultural and histologic data taken together indicated that a minimum of one third of these patients contract or activate latent cytomegalovirus infections during the first few months after engraftment; and patients with marked rises in complement-fixing antibody titer either had no clinical manifestations or recovered from transient interstitial pneumonia. Most of these patients excreted cytomegalovirus in urine. In contrast, patients with fatal cytomegalovirus pneumonia rarely demonstrated a noteworthy rise in complement-fixing antibody titers and have not, in our experience, excreted cytomegalovirus in urine at the time of the disseminated infection. The implications of these findings, as well as their precise relation to immune reconstitution and other factors that might affect the pattern of response to cytomegalovirus infection in transplant recipients, deserve more thorough prospective study. Finally, very little can be said about the clinical management of interstitial pneumonia and disseminated cytomegalovirus infection. All the severely infected patients have received antibiotics and pentamidine isethionate. Cytosine arabinoside, adenine arabinoside and corticosteroids have also been used but there is no clear evidence of benefit from any of these drugs at present.

In contrast to the problems associated with cytomegalovirus infection (and whatever additional and unknown agent or agents may account for interstitial pneumonia) the experience in Seattle with other opportunistic viral infections is relatively modest. Herpes simplex virus infections of the mouth and lips are frequent complications in the first few weeks after transplantation, but, with one exception, have subsided and healed without widespread dissemination. Later, cutaneous herpes zoster occurs occasionally over the period in which cytomegalovirus infections are seen. These lesions have frequently spread over several dermatomes before subsiding. Antibody responses to these viruses have been observed. The documented incidence of P. carinii pneumonia has been referred to and, perhaps surprisingly, is quite low. Antibody responses to this organism are very rare in our population of marrow-graft recipients, with only two documented cases, both of which were not in patients with proved P. carinii pneumonia.

Immunologic Status of Long-Term Survivors

Immunologic reactivity of syngeneic marrow-transplant recipients (monozygotic twins). Three patients treated with twin marrow grafts for hematologic neoplasia have been tested for immunologic reactivity. Absolute lymphocyte counts were above 1000 per cubic millimeter in all patients within one to three months after transplantation. No consistent changes in serum immunoglobulin levels were noted. Two of the three patients — both of whom had received twin peripheral blood lymphocytes as well as marrow — exhibited normal primary and secondary antibody responses to bacteriophage OX174 at six weeks and at one year after marrow transplantation. Two patients did not exhibit cutaneous delayed hypersensitivity reactions to the usual battery of skin tests, even though one of the donors was positive to candida and mumps. The third patient became positive to candida at one year. Two patients responded to dinitrochlorobenzene at one and two years. Lymphocytes from all three were stimulated in the test
with mixed leukocyte culture by irradiated allogeneic cells and by phytohemagglutinin to an extent almost comparable to that observed with the normal twin’s lymphocytes during the first few months after marrow transplantation. The three long-term syngeneic transplant survivors studied had no serious infections. Thus, the results suggest an impressive restoration of immunologic function in the three patients. It is assumed, of course, that the immunologic system tested is totally of donor type, although no markers are available. Efforts are under way to study the immunologic reactivity of twin marrow-transplant recipients early after marrow transplantation with additional measurements of immunologic function.

Immunologic reactivity of allogeneic marrow-transplant recipients (major-histocompatibility-complex-matched donors). The recovery of immune reactivity in allogeneic chimeras would be expected to be slower than that of syngeneic chimeras owing to at least three possible mechanisms: it has been hypothesized that lymphoid depletion occurs in allogeneic chimeras as a result of destruction of donor lymphoid cells during graft-versus-host disease; allogeneic chimeras have often been treated with immunosuppressive drugs after grafting, which not only delay or prevent graft-versus-host disease but also suppress the response of chimeric lymphocytes to other antigens; and the combination of toxic conditioning regimens, post-grafting immunosuppression and graft-versus-host disease causes gut damage leading to a malabsorption syndrome, which given enough time, can lead to immunodeficiency. In general, allogeneic resident chimeras are less immunologically active than their syngeneic counterparts even if studied as late as 300 days after grafting (reviewed elsewhere). In a recent study, the immune system of random-bred canine chimeras was surveyed for periods up to eight years after grafting. After a prolonged period of decreased immune reactivity lasting for 200 to 300 days after grafting, cellular and humoral immunity in these chimeras returned to the normal range. Although there was a delayed rise in antibody titers during the primary immune response, long-term chimeras showed normal peak titers and a normal qualitative and quantitative secondary antibody response. Animals were able to live in an unprotected environment without apparent increased occurrence of infection, supporting the conclusion that, after an initial phase of impaired immune function, these canine chimeras regained normal immune reactivity.

In an initial study we described evaluation of immunologic reactivity in 10 human recipients of allogeneic marrow observed for five to 20 months after engraftment. Five patients had hematologic neoplasia, and five aplastic anemia. Serum IgG, IgA and IgM levels declined in all recipients and then returned to normal by the 100th day. Absolute lymphocyte counts were above 1000 per cubic millimeter in all patients within one to three months after grafting. The recipients showed a markedly decreased antibody response to bacilliophage Ø ×174. In contrast, several of these recipients acquired cytomegalovirus or herpes zoster infections from which they recovered, and they developed good antibody titers against these agents. Testing in mixed leukocyte culture showed that recipients of allogeneic grafts had a wide range of responsiveness, but clear stimulation was observed on nearly all occasions even within the first month of grafting. Despite this in vitro activity, the allogeneic recipients, with one exception, could not be sensitized to dimethylbenzene when tested between 28 and 365 days after grafting. We concluded that recipients of allogeneic grafts showed a long-lasting immunologic deficiency that points out the necessity for vigilance in early detection and treatment of infection and the need for additional measures to restore immunologic competence in these patients.

Malignant Transformation of Donor Cells

A mechanism of leukemic relapse that was not anticipated was the possibility of malignant transformation of the engrafted marrow. Nevertheless, there is unambiguous evidence that this event has occurred twice in leukemic marrow-graft recipients. Two female patients with childhood acute lymphoblastic leukemia received total-body irradiation as their only cytoreduction in preparation for engraftment, followed by allogeneic marrow infusion from histocompatible male siblings. Both escaped the serious complications that follow marrow transplantation and appeared to achieve normal marrow status, only to suffer a florid leukemic relapse 69 and 135 days after engraftment. In both cases careful cytogenetic study of a large number of metaphase chromosomes, and in the second case, fluorescent staining for y chromatin in interphase nuclei, indicated that the recurrent leukemia was composed of male cells and was therefore of donor origin. There are only four other patients in our series who are strictly comparable to these two—that is, children with acute lymphoblastic leukemia prepared only with total-body irradiation. Three of these have also shown leukemic relapse, but in no case was an opportunity presented to determine the host or donor origin of the recurrent leukemia. The one leukemia-free patient in this group is our longest survivor at four years after grafting.

What caused the malignant transformation of the marrow graft? Several explanations have been discussed. It has been suggested that leukemia, at least in some cases, could be a disease of "regulation" such that any marrow in a leukemic host will become leukemic. Another theory advanced involves a cell-fusion event between donor and host cells followed by a loss of extra chromosomal material or "diploidization" with retention of the y chromosome. Still another suggestion is that immunodepression in the marrow-graft recipients could conceivably lead to a breakdown in the putative immune surveillance defenses against spontaneously arising malignant cell clones in the regenerating engrafted tissue. Furthermore, it should be noted that graft-versus-host reactions are themselves leukemogenic, at least in mice. Against the last two proposals is our failure to observe malignant transformation of marrow grafts in non-leukemic recipients. In addition, leukemogenesis mediated by graft-versus-host disease in mice appears to involve activation of endogenous C-type murine leukemic viruses. This point raises a final and perhaps most plausible hypothesis—specifically, that a leukemogenic virus or similar etiologic agent
resident in a recipient infected and transformed the transplanted marrow. Ionizing radiation will induce production of occult C-type leukemia viruses in some experimental animal systems. Moreover, oncovirus infections can be transmitted by fragments of cellular DNA containing an integrated DNA provirus, at least in some systems. Thus, one need not postulate the presence of recognizable whole-virus particles, but rather could envision transmission through the agency of subviral or other oncogenic subcellular material released from the degenerating host leukemic-cell population.

This list may well not exhaust all the possibilities underlying marrow-graft transformation, but in the absence of information on the ultimate mechanism, there are a number of questions of immediate consequence. The most important of these is the frequency and prevalence of leukemic transformation. The existence of a mechanism for the frequent induction of neoplasia in new populations of cells in the course of acute leukemia and related diseases might potentially threaten the ultimate success of intensive intermittent-therapy programs dependent upon the stepwise destruction of the tumor-cell population. At present, however, there is no conclusive evidence that induction of malignant transformation in new clones of cells does occur with noteworthy frequency outside of the two cases discussed here and the observation of apparent reversion of new tumor clones in certain late relapses of African Burkitt’s lymphoma.

Adoptive Immunotherapy

The data on allogeneic marrow transplantation for hematologic neoplasia permit no conclusion about the existence or absence of an antileukemic effect of graft-versus-host disease as anticipated from animal studies. The principal evidence that graft-versus-host disease, whether or not clinically detectable, did exert an antileukemic effect stems from the observation that leukemia recurred more frequently in recipients of twin marrow than in recipients of allogeneic marrow — in which the potential for graft-versus-host disease existed. Analysis of data on the patients whose leukemia cleared from the marrow and who survived for more than 60 days reveals that 13 out of 22 such patients exhibited recurrent leukemia after twin marrow grafts whereas leukemia recurred in only 5 out of 49 patients treated with an allogeneic graft. It must be emphasized, however, that clinically detectable graft-versus-host disease does not correlate with presence or absence of recurrence of leukemia. Most importantly, clinically detectable graft-versus-host disease is not essential for long-term, complete remissions. Of nine long-term survivors free of acute lymphoblastic leukemia, seven never had clinically evident graft-versus-host disease, and neither did five of nine patients whose leukemia relapsed. Of the long-term survivors free of acute myelogenous leukemia six of 10 did have graft-versus-host disease, but so did two patients whose leukemia recurred. Thus, it is not possible to recognize an antileukemic effect of graft-versus-host disease from the data currently available.

**Current Directions of Research**

**Nature of “Tolerance”**

In a stable long-term chimera, the immunologically competent foreign graft does not mount a harmful reaction against its host. The most common explanation is that the lymphoid cell clones that would have been reactive against host alloantigens have been made "tolerant" — i.e., specifically non-reactive. Another possibility is that the reactive cell clones have been eliminated in the course of an immunologic interaction. Alternatively, a form of immunologic enhancement may be involved, by which a factor in the chimeric individual’s serum can protect against cellular immune reactions. We studied nine canine irradiation chimeras between 178 days and 7.5 years after transplantation of allogeneic marrow. The lymphocyte populations were foreign to their hosts and, therefore, theoretically capable of mounting a graft-versus-host reaction. Lymphocytes from the chimeric dogs were found to inhibit colony formation by their "own" fibroblasts, whereas lymphocytes from other chimeras or from normal dogs did not (the test used was the colony-inhibition assay). Serum from the chimeras specifically abrogated this inhibitory effect. These initial results suggested that the immunologic "tolerance" of the chimeric dog was mediated in vivo by blocking serum substances. Subsequently, similar findings were reported in rodent radiation chimeras, in mice made tolerant by neonatal injections of allogeneic lymphocytes and in tetraparental mice.

More recently we studied nine canine irradiation chimeras and their DL-A-matched marrow donors between 545 and 1226 days after marrow grafting, using a microcytotoxicity or cell-inhibition assay. Before testing, marrow donors were immunized against their chimeras by repeated skin grafts, which they rejected. Skin fibroblasts from chimeras and their donors were tested for cell inhibition by exposure to sera from lymphocytes from chimeras, donors and normal dogs. Lymphocytes from sensitized marrow donors inhibited fibroblasts from their chimeras (eight of nine dogs); cell inhibition was abrogated by chimeric serum in only three of eight cases. Only one chimera showed consistent cell inhibition of its "own" fibroblasts, and the serum did not block cell inhibition. The remaining eight chimeras did not show consistent cell inhibition. In conclusion, the cell-inhibition assay is able to detect immunity across "minor" histocompatibility barriers in dogs. Results in chimeras suggest that serum blocking factors are not a prerequisite for maintenance of stable graft-host "tolerance" in DL-A-matched recipients. Similar findings were made in a subsequent study, in which 18 canine marrow-graft recipients were studied sequentially between 50 and 450 days after grafting.

Studies are now in progress involving the use of additional test systems and involving sequential studies of individual patients starting very early after the graft in an attempt to elucidate the complex problem of the "tolerant" state in the human marrow-graft recipient.
Marrow Transplantation from Unrelated Donors

It is obvious from the clinical experiences cited above that a multitude of problems remain to be solved when marrow donor and recipient are siblings matched at the major histocompatibility complex. At the same time, since less than half the patients will have matched siblings, efforts must be directed toward identifying unrelated persons who are perfect matches at the major histocompatibility complex for the patient. With computer technology, and with a large panel of donors, matching for the serologically detected loci can now be accomplished. Rapid advances in the ability to detect and evaluate lymphocyte-determined loci are occurring at present. Thus, we can expect in the near future to be able to identify unrelated donors matched at the major histocompatibility complex, either living donors or cadaver donors whose marrow has been stored. When this goal is achieved, it is important to realize that the unrelated donor matched at the major histocompatibility complex will be no better than the sibling similarly matched. The patient will still encounter the same spectrum of problems described above for the patient with a matched sibling. Hence, it is important to continue to try to solve these clinical and immunologic problems, especially since the vast majority of patients with matched siblings are not now being considered for marrow transplantation.

DISCUSSION

It seems likely that in the near future marrow transplantation will be attempted as therapy for any variety of malignant neoplasia that is sensitive to irradiation or chemotherapy and for non-malignant disorders of the marrow such as thalassemia and sickle-cell disease. At present each new patient with acute leukemia or aplastic anemia should undergo histocompatibility testing along with the members of the family. If the patient has a sibling matched at the major histocompatibility complex, transfusion of blood products should be minimized, and transfusion from family members should be avoided. Marrow grafting can be planned at an appropriate time, perhaps immediately for patients with severe aplastic anemia or acute myelogenous leukemia but only after failure of conventional therapy for patients with mild aplastic anemia and acute lymphoblastic leukemia. Proper selection of the type of patient and the time of grafting raises too many questions to be enumerated here. For example, are the extremely refractory leukemias, described above, the result of prior intensive chemotherapy, and could this problem be avoided by earlier marrow grafting?

Allogeneic marrow grafting in man is still an exceedingly complex and difficult endeavor. Therefore, certain supportive facilities should be considered as mandatory for any center undertaking a marrow-transplantation program, including means of providing the large numbers of platelets and granulocytes that may be required, facilities for the proper administration of total-body irradiation, protection against infection, and bacteriologic monitoring. Laboratory capability should include histocompatibility typing, mixed leukocyte culture and other immuno-

logic studies, production of antithymocyte globulin, cytogenetics and blood genetic markers. The marrow-graft team, including physicians, nurses and technicians, should have experience in transplantation biology and medicine with a successful program of animal transplantation using the techniques to be applied to man. In this setting the patient should have the maximum opportunity to benefit.

Noteworthy advances in the knowledge of the cause and pathogenesis of human diseases may be expected as a corollary of marrow-transplantation studies. Examples already at hand are the demonstration, by the fact of successful engraftment, that the marrow microenvironment is normal in most patients with aplastic anemia and a demonstration that in vivo malignant transformation of normal human cells can occur in acute lymphoblastic leukemia. Marrow transplantation will also provide insights into many aspects of transplantation biology and tumor immunology. Despite the many discouraging complexities associated with marrow transplantation, some degree of satisfaction may be derived from the fact that knowledge of normal and abnormal immunology and physiology is being increased during endeavors to provide new therapeutic approaches for the patient with otherwise fatal disease.

We are indebted to Dr. Eloise Giblett, who carried out the blood genetic marker studies, and to the entire nursing staff of the Division of Oncology for their management of these patients.

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Canine models of bone marrow transplantation.

Ladiges WC, Storb R, Thomas ED.

Fred Hutchinson Cancer Research Center, Seattle, WA.

Progress in experimental bone marrow transplantation in dogs has provided for the direct transfer of research data to the clinical setting and the therapeutic application of marrow grafting to a variety of human diseases. Animal models of total body irradiation, engraftment and graft-versus-host disease are still needed to solve the existing clinical problems of marrow transplantation. Therefore, work in various canine model systems continues to be of interest. Pet dogs with spontaneously occurring lymphomas are used to study the clinical parameters necessary for applying the technique of transplanting their own marrow (autologous), in conjunction with high dose radiation and/or chemotherapy, to human patients with cancer. A major consideration in the successful transplantation of donor bone marrow (allogeneic) is overcoming histocompatibility barriers to assure engraftment and the prevention of graft-versus-host disease, a major limiting aspect of clinical marrow transplantation. Chemicals, radiation, radiotherapeutic techniques, antisera and monoclonal antibodies have been and continue to be developed in laboratory bred dogs. These approaches suppress the immune system either nonspecifically by ablation of immune reactive tissue, or specifically by affecting certain types of immune reactive cells. Parameters such as clinical effectiveness (engraftment or prevention of graft-versus-host disease), immune reconstitution and undesirable side effects in long-term survivors are all used to determine whether new technology can be transferred from preclinical canine studies to human bone marrow transplantation protocols.

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Fatty Acid Composition of Adipose Cells in Red and Yellow Marrow: A Possible Determinant of Haematopoietic Potential

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The fatty acid composition of whole bone marrow and that of isolated, disaggregated adipose cells from red and yellow marrow was examined by gas chromatography. Consistent and significant shifts from myristic and palmitic acids (in red marrow) to their respective monounsaturated derivatives myristoleic and palmitoleic acids (in yellow marrow) were found. These differences in the fatty acids correlate with histochemical studies and lend further support to the concept that the composition of lipid in the adipose cells of bone marrow may determine their relative stability in relation to haematopoietic requirements.

Key words: adipocytes – bone marrow – fatty acid composition – haematopoietic potential

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Man, like some of the other mammals, has areas of haematopoiesis unevenly distributed throughout the medullary cavity. In peripheral sites only adipose tissue or yellow marrow occurs and this seldom participates in blood formation even when demands are increased (Maniatis et al 1971, Tavassoli et al 1974). This situation contrasts with the more centrally located red marrow where the fat cells are intimately associated with haematopoietic elements and here they are in a state of flux being readily replaced by expansion of blood-forming tissue (Tavassoli et al 1972).

This striking difference in the physiology of marrow fat cells has not been elucidated, but there is evidence to suggest that those associated with the red marrow are functionally and structurally distinct from the morphologically similar cells comprising yellow marrow. For example, Cohen & Gardner (1965) reported that the yellow marrow partially defends itself against starvation whereas fat in red marrow is readily mobilized in parallel with the extramedullary adipose tissues. We have confirmed these findings (Tavassoli 1974a) and extended them with the observation that
phenylhydrazine-induced haemolysis leads to resorption of lipid contained in fat cells of red marrow whereas the yellow marrow is relatively stable (Tavassoli et al 1972, Tavassoli 1976).

Furthermore, in the course of histochemical studies of bone marrow, it was noted that the lipid substance contained within the fat cells of red marrow stained with performic acid-Schiff (PFAS), whereas those in yellow marrow did not. Significantly, when haematopoietic tissue hypertrophied in response to experimentally induced haemolysis, the former resorbed while the latter remained unchanged (Tavassoli 1976). These clear differences in the lipid composition of fat cells or adipocytes in the 2 types of marrow may account for their contrasting behaviour and, therefore, more precise characterisation was undertaken using gas chromatography. Since the bulk of this material has been shown both biochemically and histochemically to be triglyceride (Lund et al 1962, Tavassoli 1974) analysis was restricted to the fatty acids.

MATERIALS AND METHODS

Materials. New Zealand white rabbits, 10 to 12 months old, were used in the study. The sternum, 3 thoracic vertebrae, one or both femora, the tibias and os calcis were removed, weighed, broken into small pieces and separately homogenized in a blender. Vertebræ were taken as representative red marrow and os calcis as yellow marrow. Samples were also processed directly for lipid analysis.

Preparation of adipose cells. The adipose cells are disaggregated and separated from haematopoietic and stromal elements of the bone marrow after Rodbell (1964) and Adebonojo (1975). The bone marrow is sliced into small pieces and placed in 10 ml of McCoy's medium in a 25 ml siliconized Erlenmeyer flask to which is added 10 mg of collagenase for each g of tissue. The mixture is incubated at 37°C with slow constant gyratory rotation (50-100 rpm) for 1 h. Thereafter the contents of the flasks are gently stirred, the cells dispersed with a plastic spatula and any residual pieces of tissue are removed before centrifuging the suspension at 400 g. Fat cells and droplets of fat ruptured cells float to the surface forming a layer which is decanted, resuspended in fresh medium and again centrifuged. The latter step is repeated, after which the cells were plated in monolayer using 25 ml plastic culture flasks and incubated at 37°C during which time the adipocytes adhere to the bottom of the flasks. After 4 h the medium was changed and the cells were removed using EDTA and trypsin. Microscopic examination of these preparations, stained with oil Red O, confirmed them to be a pure preparation of adipocytes containing lipid vacuoles. Since individual preparations did not yield sufficient material for analysis, this was carried out on pooled samples.

Extraction of fatty acids for gas chromatography. The lipid fraction was extracted into chloroform (Hammarstrand 1966) by adding 20 ml of a chloroform-methanol mixture (2:1 by volume) to each g of tissue in the homogenate. Protein precipitate was removed by filtration through coarse fat-free filter paper and saline added to the extract in an amount equivalent to ¼ of the extract volume. The mixture was then vigorously shaken and allowed to stand overnight. The following day the upper aqeous phase was discarded and the lower chloroform phase, which contained the lipid, was evaporated to dryness under a stream of nitrogen and the residue containing the total fraction was weighed.

This residue was saponified by adding 50 ml of ethanol-ethyl ether (3:1 by volume) and 0.5 ml of 10 N KOH and allowing it to stand in a boiling water bath for 2 h. Sufficient water was then added to the soap solution to obtain a 50% ethanol-water solution of soap, after which 75 ml of petroleum ether were added and the solution was allowed to stand overnight. The following day the upper phase was discarded and the free fatty acids were liberated from the lower phase with 10 ml 0.5 N HCl, and these were extracted into a further 75 ml of petroleum ether.

Free fatty acids were methylated by the addition of 0.5 ml H2SO4 in 10 ml methanol and the esters extracted in 30 ml petroleum ether. The ex-
tract was evaporated to dryness below 40°C under nitrogen to prevent evaporation of the short chain methyl esters. The dry residue, a measure of total fatty acids, was weighed. The distribution of the various fatty acids was studied in a Varian Aerograph using a 12" x 1/8" stainless steel column packed with 20% diethylglycosuccinate on Chromosorb W, 60/80 mesh at a temperature of 205°C. The carrier gas was N₂ at a flow rate of 25 ml per min. All reagents were of analytical grade.

RESULTS

Terminology. In bone marrow, the bulk of fat is contained within the adipocytes which have a diameter between 140–160 μm, most of which is occupied by a single fat globule and several smaller ones (Tavassoli 1974). Lipid analysis (Lund et al 1962, Tavassoli 1974) has demonstrated that fat globules consist almost entirely of neutral fat and

Figure 1. Representative chromatograms for fatty acids of isolated, disaggregated marrow adipose cells from (A) vertebrae and (B) os calcis. Vertebral marrow is representative of red marrow while that of os calcis is representative of yellow marrow. Note the shift from palmitate (in red marrow) to palmitoleate (in yellow marrow); the pattern in tibial marrow is intermediate. Similar shifts can also be seen in myristate and myristoleate.

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<td>1.5</td>
<td>1.4</td>
<td>18.1</td>
</tr>
<tr>
<td>9</td>
<td>1.1</td>
<td></td>
<td>15.9</td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>1.5 (± 0.3)*</td>
<td>1.2 (± 0.3)*</td>
<td>17.6 (± 0.6)**</td>
</tr>
<tr>
<td>Os Calcis adipose cells</td>
<td>2.3</td>
<td>1.1</td>
<td>26.8</td>
</tr>
<tr>
<td>Femur</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (± SD)</td>
<td>1.7 (± 0.3)</td>
<td>0.8 (± 0.07)</td>
<td>26.3 (± 2.0)</td>
</tr>
<tr>
<td>Femoral adipose cells</td>
<td>2.2</td>
<td>0.4</td>
<td>28.1</td>
</tr>
</tbody>
</table>

* Differences highly significant at p < 0.001  
** Differences highly significant at p < 0.001  
*** Differences not significant

Free fatty acids: phospholipids are absent. In adult rabbits nearly half the volume of marrow in the axial skeleton (ribs, vertebrae and sternum) consists of fat, that of the os calcis almost entirely of adipocytes with the tibial and femoral marrow being intermediate (Tavassoli 1974, 1976).

**Lipid analysis.** The data for both whole marrow and isolated adipocytes from the 3 representative areas are set out in the table and figure.

Firstly, it can be seen that gas chromatographic analysis exposes distinct differences in the pattern of the individual fatty acids depending on the area from which the material has been collected. The proportion of myristate decreases gradually as the areas of yellow marrow are approached, with the highest levels being present in vertebrae and
and in marrow adipose cells from different rabbit bones

<table>
<thead>
<tr>
<th>Palmitoleate 16:1</th>
<th>Stearate 18:0</th>
<th>Oleate 18:1</th>
<th>Linoleate 18:2</th>
<th>Total of fatty acid % total lipid</th>
<th>Saturation index</th>
</tr>
</thead>
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<tr>
<td>3.5</td>
<td>33.9</td>
<td>22.5</td>
<td>5.2</td>
<td></td>
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</tr>
<tr>
<td>3.1</td>
<td>34.3</td>
<td>23.4</td>
<td>5.6</td>
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</tr>
<tr>
<td>3.5</td>
<td>36.5</td>
<td>21.4</td>
<td>5.6</td>
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<tr>
<td>4.0</td>
<td>36.1</td>
<td>27.9</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**3.4 (± 0.2)**       **34.9 (± 1.0)** **23.4 (± 1.9)*** **5.6 (± 0.2)*** **9.3** **1.4**

1.4 **29.3** **23.6** **2.4** **27.5** **2.6**

| 14.0 | 36.6 | 22.4 | 5.8 |
| 14.3 | 35.8 | 22.8 | 6.0 |
| 15.0 | 37.1 | 21.9 | 5.6 |
| 15.2 | 35.2 | 23.9 | 6.2 |
| 13.6 | 37.2 | 23.0 | 5.6 |
| 15.0 | 36.2 | 21.4 | 5.8 |
| 13.9 | 34.8 | 25.3 | 6.2 |
| 15.0 | 28.5 | 19.5 | 6.0 |
| 13.7 | 35.3 | 28.8 | 5.2 |

**14.4 (± 0.6)**       **35.1 (± 2.6)** **23.2 (± 2.6)*** **5.8 (± 0.3)*** **60.3** **1.1**

14.3 **24.3** **26.6** **3.7** **55.2** **1.2**

| 5.4 (± 1.0) | 29.5 (± 1.1) | 25.7 (± 0.9) | 6.3 (± 0.9) | 20.5 | 1.4 |
| 3.3         | 27.8          | 32.9          | 4.5          | 38.1 | 1.4 |

s sternum and lowest in the os calcis with intermediate figures obtained for femur and tibia. By contrast myristoleate increases from vertebrae towards os calcis. A similar but much more dramatic shift from palmitate to palmitoleate is evident and the prominence of this change reflects the greater contribution of palmitate to total fatty acid pool.

A comparable pattern is evident when isolated adipose cells are examined with a consistent shift from myristate and palmitate in vertebrae to their monounsaturated derivatives (myristoleate and palmitoleate respectively) in os calcis. These differences are statistically all highly significant.

The bulk of the fatty acids in both red and yellow marrow consists of stearate and oleate which, together, account for nearly half of the bone marrow fatty acids. There was no apparent difference in the proportion of these two substances in red and yellow marrow when whole tissue is studied. However, the data from isolated adipose
cells suggest a shift from stearate in vertebral adipocytes to oleate in those from the os calcis.

Furthermore, it is clear that the fatty acid fraction of total lipid is lowest in the vertebral marrow and highest in that from the os calcis with intermediate values being obtained for femur and tibia. This gradient parallels both the proportion of fat cells and the total fat content within the respective bones and is inverse to that of the blood forming elements, suggesting that the fatty acid content of marrow is contributed largely by the adipocytes.

Finally, the ratio of saturated to unsaturated fatty acids is somewhat higher in the red when compared to the yellow marrow.

DISCUSSION

The bone marrow is a dynamic organ normally capable of rapidly changing its fat content in response to expansion or contraction of the haematopoietic mass. That 2 apparently distinct types of fat cells can be identified and that differences in their composition can be related to the kinetics of haematopoiesis, suggest an important biological role for the adipocyte. This concept is supported by our observation that clear and consistent variations exist between the fatty acid composition of red and yellow marrow and, furthermore, that these reside in the adipose cells of this organ. Earlier histochemical studies (Tavassoli 1976) correlating areas of haematopoiesis with PFAS-positive cells are now given quantitative expression by chromatographic fatty acid profiles documenting a constant shift from myristate and palmitate in the red marrow to their monounsaturated analogues in yellow marrow. While acknowledging that the dissimilarities demonstrated in this and previous studies (Cohen & Gardner 1965, Tavassoli et al 1974, Tavassoli 1976) are not necessarily related to the functional differences between red and yellow marrow, the close correlation of fatty acid profile to the extent of haematopoietic activity strongly suggest a causal relationship. Furthermore, other works (Snyder 1965, Levis et al 1975) have also reported experimental studies in which the lipid composition of marrow is altered as medullary haematopoiesis changes.

We advance the hypothesis that the composition of lipid in the adipose cells of the bone marrow may determine their relative stability in relation to haematopoietic requirement. These changes can be reconciled with suggestions that differential blood formation in red and yellow marrow may relate to variations in temperature between the bones (Huggins & Blockso 1936, Weiss & Wislocki 1956, Petrakis 1965, Van Dyke 1967, Tavassoli 1970) by postulating that this is mediated through the lipids of the marrow. For example, a temperature gradient may alter lipid composition which could then modulate the potential of marrow for replacement of fat cells by haematopoietic elements. Additional support for the importance of bone marrow lipid structure in the regulation of haematopoietic cell proliferation derives from recent studies that these substances are implicated as bioregulators of cell growth and proliferation (Holley et al 1974, Inbar & Schinitzky 1974, Leder & Leder 1975).

We conclude that the striking and reproducible differences that exist between the fatty composition of red and yellow marrow support the concept that these substances within the adipocytes are important factors in determining the ability and the extent of the marrow contained in different bones to
respond to the expansion of blood forming elements under physiologic circumstances.

ACKNOWLEDGEMENTS

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MEDICAL PROGRESS

BONE-MARROW TRANSPLANTATION

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Alexander Fefer, M.D., F. Leonard Johnson, M.B., B.S., Paul E. Neiman, M.D.,
Kenneth G. Lerner, M.D., Harold Glucksberg, M.D., and C. Dean Buckner, M.D.

The modern era of bone-marrow transplantation was ushered in by the experiments of Jacobsen, Lorenz and their colleagues, who showed that mice could be protected against otherwise lethal irradiation by shielding of the spleen or by intravenous infusion of marrow. At first it was thought that this protective effect was due to a humoral factor. By 1956, however, several laboratories, using a variety of blood genetic markers, demonstrated that the protective effect against lethal irradiation was due to the colonization of the recipient marrow by donor cells.

An article on clinical marrow transplantation that appeared in this journal showed that large amounts of marrow could be infused intravenously with safety, and described a transient marrow graft in man. It also provided estimates of the number of marrow cells needed and pointed out the potential application of marrow grafting to radiation accident victims, to the therapy of leukemia and to the therapy of patients with immunologic deficiencies (at that time collected under the heading of agammaglobulinemia). In the following year, Mathé et al. attempted the dramatic treatment, by marrow transplantation, of six human victims of an irradiation accident. Despite the promising potential usefulness of marrow transplantation, the next decade was one of frustration and disappointment. Most marrow grafts were carried out in terminally ill patients who did not live long enough for a graft to be evaluated. The few successful allogeneic grafts were followed by a lethal immunologic reaction of the graft against the host.

Recent advances in the knowledge of histocompatibility typing, in the prevention and management of graft-versus-host disease and in supportive measures for patients with no marrow function have renewed interest in the subject of marrow transplantation. In this article we shall attempt to review the basic immunobiology relating to and derived from the field of marrow transplantation and to describe the clinical progress that has been made, recognizing that this is only the beginning of a new phase in the development of a medical therapy.

Experimental Background

Studies in Rodents

The availability of inbred strains of mice has made possible extensive study of the genetic systems that govern acceptance or rejection of a tissue graft. The most widely studied immunosuppressive regimens to prepare the recipient for allogeneic marrow engraftment in rodents have been total-body irradiation or cyclophosphamide (or both), with occasional addition of antilymphocyte serum. The amount of irradiation required to permit engraftment and prevent rejection of incompatible marrow is higher than that which will induce fatal marrow aplasia. Total-body irradiation is most immunosuppressive if administered within 24 hours before the antigen, regardless of the nature of the antigen. Cyclophosphamide is an effective immunosuppressant in nonlethal doses. It is effective when given one day before the allogeneic marrow, but is more suppressive if administered one day after exposure to tissue antigens of the marrow donor. Although cyclophosphamide can suppress an established cell-mediated immune response against some
antigens in some animal models, it did not permit marrow engraftment in a rodent host presensitized to donor antigens several days before the cyclophosphamide administration and marrow infusion.

Graft-versus-host disease occurs whenever marrow or other tissue containing immunologically competent allogeneic cells is infused into a host who is unable to reject the infused donor cells. The pathophysiology remains unclear, especially the contribution of the host to the disease. The effector cell mediating graft-versus-host disease is probably a T cell, but involvement of other cell populations is likely. Although rodents with graft-versus-host disease die of infection that is presumably due to the immunoincompetence caused by the disease, it is possible that infection is an intrinsic part of the graft-versus-host disease per se with activation of latent infectious agents.

The treatment of graft-versus-host disease is a formidable problem. Methotrexate has been effective prophylactically. Cyclophosphamide, procarbazine, the combination of cyclophosphamide and prednisolone, and cyclosporine arabinoside are somewhat effective even against established graft-versus-host disease in some rodent models. Other approaches reported to have some effect in some models included administration of host lymphoid cells and antibody directed against donor isoantigens or against host isoantigens (as a form of enhancement). To date, the most impressive therapeutic results have been reported in mice kept in a germ-free environment and prepared with lethal total-body irradiation. When the animals were given allogeneic marrow the rate of fatal graft-versus-host disease markedly decreased, but spleen cells still resulted in fatal graft-versus-host disease. Finally, the literature is replete with approaches to specific or preferential elimination of lymphoid cells capable of inducing graft-versus-host disease from donor marrow suspensions. These approaches remain highly experimental and variably efficacious.

The application of marrow transplantation to tumor therapy received early attention on the basis of the rationale that tumors, especially hematologic neoplasms, might be eradicated by lethal doses of total-body irradiation and that donor marrow would repopulate the host and prevent radiation-induced death. The attempts to use such an approach have been based largely on studies in readily manipulable rodents bearing a variety of tumors as recently reviewed. Interpretation of the results of syngeneic marrow transplantation in tumor-bearing mice has been influenced by several findings in the area of tumor immunology: most murine tumors possess tumor-associated antigens capable of evoking a cell-mediated as well as humoral antibody response against them; adoptively transferred lymphoid cells can inhibit tumor growth but only if the cells are immune to tumor-associated antigens, but with rare exceptions, such adoptive tumor immunotherapy, although characterized by unique antitumor specificity, can cope with only a small tumor load; and in several murine tumor models such syngeneic adoptive immunotherapy when used as an adjunct to sublethal chemotherapy can eradicate even clinically evident leukemia if the syngeneic lymphoid cells are immune to tumor-associated antigens.

Allogeneic marrow transplantation for leukemia represents a final common pathway between the problems of transplantation immunology posed by the graft and the problem of antitumor chemotherapy and immunotherapy posed by the presence of leukemia. The only additional element introduced is that of the effect of graft-versus-host disease on the leukemia and possibly the effect of the leukemia on the severity or expression of the graft-versus-host disease. Barnes et al. postulated that allogeneic marrow infused into leukemic mice after lethal total-body irradiation would colonize the host and would "destroy, by the action of the immunity these residual leukemic cells — and perhaps also the host." Many studies with histoincompatible cells have shown that graft-versus-host disease when sufficiently severe will affect some tumors, but most mice will die of graft-versus-host disease whereas a few mice will survive the fatal disease and will be tumor free. Mathé suggested the possibility that one might use the graft-versus-host disease against the tumor, but then treat the graft-versus-host disease and save the cured host. Unfortunately it is extremely difficult to control the graft-versus-host disease once it is established, even in non-tumor-bearing mice. One approach was recently illustrated in a murine tumor model in which nonlethal subclinical graft-versus-host disease was effective against a disseminated leukemia, but the same reaction was immunotherapeutically more effective when induced by lymphoid cells from donors preimmunized against tumor-associated antigens.

Studies in Dogs

In our laboratory we have carried out extensive studies with dogs, as an animal model of random-bred species, for studies of principles and techniques applicable to man. Dogs have a major histocompatibility complex called DL-A (reviewed by Albert et al.). Canine families are readily available for genetic studies of transplantation antigens, and canine litters provide matched sibling pairs simulating the HL-A-matched human sibling pairs. Important observations include the following:

1. Studies in dogs indicated that cyclophosphamide can be substituted for total-body irradiation to condition recipients for marrow transplantation.

2. Marrow can be effectively stored at low temperatures in dimethyl sulfoxide for use in marrow grafting.

3. Stem cells in the bulky coat from the peripheral blood are able to repopulate the irradiated marrow spaces. Such stem cells are absent among thoracic duct lymphocytes.

4. The dog was the first animal in which the predictive value of in vitro histocompatibility testing for the outcome of marrow grafts was demonstrated. Canine littermates matched at the major histocompatibility complex survive better than mismatched ones, but approximately 50 percent of the animals succumb to late graft-versus-host disease. This result indicates that "minor"histocompatibility differences also can play a part in the development of fatal graft-versus-host disease. Such disease can be pre-
vented or reduced in severity by the prophylactic use of methotrexate after grafting. The drug can be discontinued approximately three months after transplantation without subsequent development of graft-versus-host disease. Antithymocyte serum is of value in treating established graft-versus-host disease.

5. Blood transfusions before grafting, even from a donor compatible at the major histocompatibility complex, may sensitize the intended marrow-graft recipient and make graft rejection much more likely to occur.

6. Long-term healthy marrow chimeras can be achieved in this random-bred species with chimerism persisting for at least 10 years. These animals can be used in studies on the nature of the operational "tolerance" necessary to maintain the stable chimeric state.

7. Certain canine spontaneous diseases such as hemophilia, cyclic neoplasia, malignant lymphoma, leukemia and other malignant solid tumors as well as hemolytic anemia associated with congenital pyruvate kinase deficiency are valuable models to study the use of marrow grafting for the treatment of these diseases. For instance, it was possible to show that canine cyclic neutropenia is not due to a deficiency of marrow regulation, but rather to a stem-cell defect that can be corrected by marrow transplantation.

In canine hemophilia, orthotopic transplantation of a normal liver into a hemophilic dog resulted in complete correction of the deficiency of factor VIII. However, there are noteworthy extrahepatic sources of factor VIII, since hepatotransplanted normal dogs bearing a transplanted liver from a hemophilic dog show factor VIII levels equivalent to that seen in the heterozygous state. Marrow grafting studies ruled out the hemopoietic and lymphoid systems as sources of factor VIII production.

Studies in Nonhuman Primates

It was thought that the monkey was the animal of choice for study of problems in marrow transplantation because of its close phylogenetic relation to man. The work of the Dutch researchers has focused on the early occurrence of fatal graft-versus-host disease in the monkey after transplants between randomly selected donor and recipient.

It was believed that the monkey differed in that respect from the dog and mimicked more closely the human situation, in which rapid onset of graft-versus-host disease with fatal outcome had been reported. When dog and monkey were compared, however, severity of graft-versus-host disease, time of onset and fatality rate did not seem to be different if donor and recipient were known to differ at the major histocompatibility complex. Van Bekkum and DeVries have provided excellent descriptions of the histopathology of the lesions of graft-versus-host disease observed in the monkey. Other studies in primates have focused on conditioning regimens. It was observed that very high doses of cyclophosphamide are needed to obtain successful grafts of allogeneic marrow. It was also found that the limiting toxicity of the antineoplastic drug in the primate is fatal cardiac toxicity. The problem with the monkey model is related to the fact that the animals are scarce, expensive and breed slowly. For these reasons, studies on grafts between monkey siblings have not yet been reported. The monkey is difficult to handle during the necessary intensive-care post-grafting. Also, monkeys with spontaneous hematologic and neoplastic diseases are rarely available.

HUMAN MARROW TRANSPLANTATION

Histocompatibility

Syngeneic or isogeneic marrow grafts, as between inbred mice or between identical twins, involve donors and recipients carrying the same tissue antigens, and thus there is no immunologic barrier to transplantation. A special kind of syngeneic graft, an autologous marrow transplantation, refers to infusion of the patient's own marrow that was set aside before intensive radiation therapy or chemotherapy.

An allogeneic marrow graft involves a donor and recipient of different genetic origin within the same species. Such transplants involve moderate to severe histoincompatibility and present a bidirectional immunologic barrier to transplantation. In the first place, the recipient may react against the graft and reject it. Secondly, a problem unique to the transplantation of tissue containing immunologically competent cells, the infused marrow cells from the donor may react against the host to produce the illness known as graft-versus-host disease.

In the mouse the major histocompatibility complex, called H-2, is composed of at least two serologically detected loci, an immune recognition locus and two lymphocyte-detected loci recognized by reactivity in mixed leukocyte culture. In addition to the H-2 region, a number of minor histocompatibility loci have been identified. The complexity of the major histocompatibility complex in the mouse seems to be reflected in the outbred species studied, principally the dog (reviewed by Albert et al.), the monkey and man.

In man, the major histocompatibility complex involves two closely associated serologically detected loci, the first, or "L.A," locus, and the second, or "four," locus. Antigens determined by the serologically detected loci are recognized by cytotoxic isoantisera raised by immunization or that arise during the course of pregnancy. More than 13 first-locus and 15 second-locus antigens can now be identified, and the resulting very large numbers of haplotypes make this the most complex genetic region yet recognized in man. The term haplotype was introduced by Cepelini to indicate the products of the major histocompatibility complex in haploid form. In addition to the serologically detected loci, there is a closely associated lymphocyte-detected locus just outside the "four" locus. Studies of the lymphocyte-detected locus are currently under way utilizing lymphocyte-detected homozygous cells as identified in the progeny of related marriages. Already several lymphocyte-detected types have been identified.

Marrow grafts between unrelated human beings carry a
high probability of major histoincompatibility due to the complex polymorphism of the major histocompatibility complex. Within a family, however, the situation is simplified considerably, since only four haplotypes can be involved (two from each parent) and since unrecognized, closely linked loci may be expected to segregate with the recognized HL-A antigens. HL-A typing of the family usually permits a genetic analysis of the four haplotypes, and offspring who have inherited the same two haplotypes are referred to as “genotypically matched.” The apparent match can be confirmed by nonreactivity in one-way mixed leukocyte culture. Some loss of potential donors occurs as a result of ABO incompatibility, although a few marrow grafts have succeeded despite ABO differences. The number of patients with matched siblings is quite large, particularly since many patients have more than one sibling. In practical terms, in the course of HL-A typing of 533 patients with at least one sibling, we have found 255 (48 per cent) who had HL-A matched siblings.

With a large “bank” of individuals of known HL-A phenotype, it is possible to identify unrelated individuals who are phenotypically HL-A matched, some of whom will not react in mixed leukocyte culture. However, in our laboratory 28 such pairs have reacted in mixed leukocyte culture, indicating that a strong incompatibility still exists. The current research on lymphocyte-detected typing, mentioned above, offers a potential solution to this problem.

Preparation of the Recipient

The recipient of a syngeneic graft requires no immunosuppressive preparation. Similarly, the patient with severe combined immunologic deficiency disease requires no immunosuppressive preparation because of the nature of his disease. All other recipients of marrow grafts must have some form of immunosuppressive preparation so that they will not reject the graft. The type of preparation is influenced by the nature of the underlying disease.

In marrow grafting for non-malignant conditions, the preparation of the recipient can be directed solely at the problem of immunosuppression without concern for the problem of eradicating malignant cells. Most of our patients with aplastic anemia have been prepared with a modification of the regimen of Santos, which involves the administration of 50 mg of cyclophosphamide per kilogram on each of four days followed 36 hours later by the marrow infusion. Cyclophosphamide is administered 24 hours after infusion of antigen from the prospective donor, usually in the form of buffy-coat cells. Nausea and vomiting accompanying administration are usually severe but transient. A high urine flow must be maintained to avoid severe cystitis due to cyclophosphamide metabolites excreted in the urine. The use of diuretics is recommended because of an antidiuretic effect of the drug. Since in aplastic anemia marrow “space” is already available, Amiel et al. conditioned seven patients with the disorder for allogeneic marrow grafting by injection of horse antilymphocyte serum. In three patients they observed engraftment of erythroid marrow, but not lymphoid cells, although the grafts generally were transient.

In malignant disorders, specifically acute leukemia, preparation of the recipient must involve not only immunosuppression but also therapy designed to kill all or nearly all of the leukemic cells. Total-body irradiation has been the most common means of conditioning a marrow-graft recipient. Studies in dogs showed that a midline tissue exposure of 500 rad of total-body irradiation, although lethal, was not sufficiently immunosuppressive to permit successful grafting of allogeneic marrow. Consistent and sustained engraftment of allogeneic marrow in the dog was achieved only when the irradiation was raised to 950 rad. Canine radiation chimeras have always been “complete” chimeras — i.e., analyses of karyotypes of cells in peripheral blood, marrow and lymph nodes up to eight years after marrow grafting have consistently shown only cells with donor karyotype — whereas dogs prepared with cyclophosphamide were “mixed” chimeras — i.e., both donor and host cells were present. The finding of persisting host cells indicates that cyclophosphamide may be less desirable than total-body irradiation in efforts to treat hematopoietic neoplasias by marrow grafting.

In preparing leukemic patients for grafting, we have routinely administered a 1000-rad midline tissue dose of total-body irradiation. Opposing 60Co sources are used in an effort to achieve more homogenous irradiation. Rotating the patient to achieve homogeneity not only is cumbersome but may be undesirable when one is dealing with a migrating population of malignant cells (leukemia). Acceptable homogeneity might be achieved with a single very high energy source but successful marrow grafts in large animals have not been reported with those technics. The acute nonmarrow toxicity is generally well tolerated, consisting of low-grade fever, immediate but transient nausea, vomiting and diarrhea and tender swelling of the parotid gland that resolves within 24 to 48 hours.

Leukemic cells are sensitive to irradiation; irradiation penetrates to all “privileged” sites and is an effective immunosuppressive agent. However, when we used irradiation only, we observed a high rate of recurrent leukemia. To increase the leukemic-cell kill we decided to give cyclophosphamide, 60 mg per kilogram, five and six days before irradiation. The result has been a striking decrease in the rate of recurrent leukemia. In the present series of patients, we are administering additional antileukemic therapy just before the administration of cyclophosphamide and irradiation with the intent of obtaining maximum leukemic cytociduction within the range of tolerable nonmarrow toxicity.

Graw et al. prepared patients with acute leukemia for engraftment by the administration of 45 mg of cyclophosphamide per kilogram given on each of four successive days. They observed early recurrence of leukemia. Santos et al. prepared leukemic patients with 50 mg of cyclophosphamide per kilogram given on each of four days. They observed the complete disappearance of host-type cells in the first few weeks after grafting. Unfortunately, most of their patients did not live long enough for complete evaluation, but one patient did have a remission of 11 months’ duration before relapse with host-type leukemic cells. More recently, Graw and his colleagues have.
prepared four leukemic patients for engraftment with a four-day drug regimen involving high doses of cyclophosphamide, cytosine arabinoside, 6-thioguanine and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). One of their patients with acute myelogenous leukemia is still in complete remission with a successful graft more than two years after grafting, suggesting that it may be possible to eradicate a leukemic cell population with drugs without the use of irradiation. Gengozian et al. prepared two patients with acute myelogenous leukemia with antithymocyte globulin and 375-rad total-body irradiation. They observed successful engraftment, indicating the adequacy of the immunosuppressive regimen and apparent absence of graft-versus-host disease, but one patient died of infection and the other of an early return of leukemia.

**Technic of Marrow Transplantation**

In the laboratory, marrow has been administered by a variety of routes, including intravenous, intra-arterial, intraperitoneal and intramedullary. VanBekkum studied the problem of the route of administration in the mouse and found the intravenous route to be best since the intraperitoneal route required about 70 times as much marrow for successful engraftment. Recent studies of this problem in the monkey showed that two to three times as much marrow is required when it is given intraperitoneally as compared to intravenously. Although some patients with immunologic deficiency disorders have had successful engraftment after intraperitoneal administration, most investigators now use the intravenous route.

Histo compatible donors are most likely to be found among members of a patient's family, and, thus, living volunteers are at present the principal source of donors for marrow grafts. One unit of blood is obtained from the marrow donor a few days before the procedure, stored in acid citrate dextrose, and returned to the donor during the aspiration, thus avoiding exposure of the normal donor to the risks of blood transfusion from another person. Marrow is obtained by multiple aspirations from the iliac bones in an operating room under sterile conditions. Spinal anesthesia is preferred, but occasionally general anesthesia is indicated. When the needle point is inserted into the marrow cavity, vigorous suction is applied while the needle is rotated. The volume aspirated from each site is limited to 1 to 3 ml to minimize dilution with peripheral blood. Marrow is aspirated and transferred quickly into a beaker containing tissue-culture medium and heparin without preservative. The pooled aspirated marrow, usually 400 to 800 ml, is passed successively through stainless-steel screens of 0.3-mm and then 0.2-mm opening. Screening produces a single-cell suspension permitting accurate cell counts and, most importantly, avoids potentially lethal marrow emboli. Cooling the marrow suspension is unnecessary and may lead to clumping of fat. The recipient must be monitored carefully during the intravenous infusion. Signs of fluid overload or strain on the right side of the heart dictate slowing of the infusion or, occasionally, phlebotomy before the infusion is completed. We have carried out marrow aspirations on more than 200 donors. Invariably, the procedure has been well tolerated. No long lasting effects are to be expected from the removal of a small fraction of a rapidly replicating tissue. The risk of anesthesia remains a legitimate concern.

**Supportive Care**

Before grafting, almost all marrow-graft recipients go through a period of no marrow function owing to their disease or to preparation for engraftment. After grafting, there is a period of 10 to 20 days before the graft begins to function. Effective supportive measures during these periods of marrow aplasia are absolutely essential to success in a marrow-transplantation program.

Before transplantation it is very important that blood products from family members should not be administered to potential recipients of sibling marrow transplants because of possible sensitization against non-HLA tissue antigens of the donor. An exception is that children of the prospective recipient may be used when the donor is to be a sibling of the recipient. After the marrow graft the family may be used as blood-product donors. The intensive use of one or two donors greatly reduces the potential exposure to cytomegalovirus, toxoplasma and hepatitis. All blood products infused into recipients of allogeneic marrow transplants are exposed to 1500 R of 60Co irradiation to prevent the proliferation of lymphoid cells, which might produce or enhance graft-versus-host disease.

Our policy is to attempt to maintain a circulating platelet count of greater than 20,000 per cubic millimeter. Platelet consumption due to infection or the underlying disease is a frequent problem. Platelets from HL-A-matched siblings are probably not subject to immunologic destruction.

Available technics do not provide the same transfusion support capability for granulocytes as for platelets. Even small elevations of circulating granulocyte levels require very large numbers of infected cells owing to their dilution in the marginal pool, the short half-life (six hours) of normal granulocytes and their rapid utilization in the face of infection. Most groups involved in granulocyte transfusion therapy of infected agranulocytic patients have concluded that such transfusions are beneficial, but objective data supporting these conclusions are understandably scarce. The largest controlled studies were conducted by Graw and McCredie and their associates. If recipient sensitization to allogeneic granulocytes is suspected, the suitability of a donor can be assessed by detection of the survival of the potential donor's platelets. For our patients the granulocyte donor has usually been the marrow donor or another family member. Such donors are well motivated to tolerate the daily inconvenience. We initiate granulocyte transfusion therapy when a serious bacterial or fungal infection develops in an agranulocytic transplant recipient; once started, the transfusions are continued daily until the recipient is able to support levels of circulating granulocytes higher than can be achieved by granulocyte transfusion. We are investigating the potential benefits of prophylactic granulocyte transfusion for uninfected agranulocytic patients in a controlled study.

We harvest granulocytes with two National Cancer Institute-IBM continuous-flow cell separators using arteriovenous shunts in the donor. This procedure is well tolerated by the donor and permits the processing of...
larger quantities of blood on a daily basis. Approximately 18 ± 8.7 (± S.D.) × 10^9 granulocytes can be collected daily by this technic. The granulocyte-rich buffy coat often contains sufficient platelets to satisfy the platelet requirements of the recipient. Reversible leukodetachment with use of Leukopaks was developed by Djerassi. Equipment for this procedure is commercially available and relatively inexpensive. There are reports of severe recipient reactions to Leukopak-collected granulocytes, but this has not been a problem in our experience.

Because of the susceptibility to infection, it seems reasonable to the patient from possible contact with pathogenic micro-organisms. Recent developments in techniques of laminar-air-flow isolation and of suppression of the gastrointestinal flora with nonabsorbable antibiotics have increased the ability to achieve the "sterile" patient. The observation that the clinical manifestations of graft-versus-host disease can be less severe in neutropenic mice than in normal mice has prompted the speculation that attempts to render human recipients of marrow transplants bacteria-free might reduce the frequency and severity of this complication. These procedures are complicated and expensive and involve patient discomfort and inconvenience. It is therefore very important that their benefits be accurately evaluated. Controlled studies of this problem are under way in our center, but no firm conclusions are available at present. As a practical matter, almost all our marrow transplants have been carried out with simple mask reverse isolation. In the absence of controlled studies, the published suggestion that marrow grafts should only be done in a sterile environment should be regarded as speculative.

**Clinical Results of Marrow Transplantation**

**Immunodeficiency**

This review is concerned primarily with marrow transplantation in persons who have achieved immunologic maturity. Studies of marrow and thymus transplantation in patients with immunologic deficiency have revealed important basic information, despite the rarity of these disorders. Details of these studies are provided in review articles. In reading these reviews, one must keep in mind that patients with severe combined immunologic deficiency are different from the cases in this report in two important respects: the disease represents an experiment of nature in which the recipient does not require immunosuppression to accept a graft; and because some myeloid marrow function is usually present, rapid marrow engraftment is not mandatory, permitting the use of very small numbers of marrow cells, given repeatedly if necessary. These differences permit the design of highly informative experiments that are not possible in patients with aplastic anemia or leukemia.

**Aplastic Anemia**

Syngeneic (monozygotic twin) transplants. Despite introduction of broad-spectrum antibiotics, androgenic steroids, corticosteroids, and support with cellular blood elements, the mortality in severe cases of aplastic anemia is 50 to 90 per cent, with many patients dying within the first three months of diagnosis. Treatment of this illness by infusion of marrow cells from a normal identical twin is of great interest since the fate of the infused hemopoietic cells might provide valuable information about the cause and the pathogenesis of aplastic anemia. Up to the present, 10 cases have been described involving patients with acquired aplastic anemia whose identical twins donated marrow. Two patients died within a few hours or days of marrow infusion. In five of the remaining eight, signs of beginning marrow regeneration were observed one to two weeks after grafting, and full recovery followed. The three patients treated in Seattle are alive and well with normal hemopoietic function six to 12 years later. Although proof of engraftment cannot be obtained in the syngeneic situation, these observations suggest that most cases of aplastic anemia are due to a persistent abnormality induced in the stem-cell population. Otherwise, engraftment of normal, syngeneic stem cells would not be expected to correct the abnormality. The patient with marrow failure who is fortunate enough to have a normal identical twin is a natural candidate for marrow transplantation and should be treated promptly since this form of therapy is essentially devoid of risk and has a high probability of success.

**Allogeneic transplants.** Through the end of February, 1974, we had treated 37 patients with aplastic anemia with transplants of marrow from major-histocompatibility-complex-matched siblings. Briefly, the patients' ages ranged from three to 67 years, with a median of 19. Twenty-three of the 37 had aplastic anemia of unknown cause: in seven the disease was associated with drugs, and in four with hepatitis; in two it occurred after a prolonged period of acquired paroxysmal nocturnal hemoglobinuria and in one it was associated with Fanconi's syndrome. The duration of the anemia ranged from one to 53 months, with a median of three months. Thirty-one patients had received therapy with androgenic steroids and had failed to respond. All but two had received multiple transfusions from unrelated donors before transplantation. Eight patients had received transfusions from parents or siblings. Eighteen patients were refractory to random platelet transfusions. Seventeen were infected at the time of admission. Granulocyte levels at admission ranged from 0 to 1000 and platelet counts from 1000 to 22,000 per cubic millimeter. Thirty-six patients were estimated to have less than 10 per cent normal megakaryocyte numbers in the marrow, 50 had less than 10 per cent granulocyte precursors, and 29 had less than 10 per cent erythroblast precursors at the time of admission.

Twenty-eight patients were conditioned for marrow grafting by administration of cyclophosphamide, 50 mg per kilogram, intravenously on each of four successive days. Nine patients were conditioned by a 1,000-rad midline tissue exposure of total-body irradiation. The day of marrow infusion was designated "day 0." After marrow grafting, methotrexate was administered intermittently as described below. Survival curves for these patients are shown in Figure 1.

Three patients died too early to evaluate success or failure of marrow grafting: one died on the day of grafting of congestive heart failure, possibly related to cyclophos-
phamide cardiac toxicity; two died on the sixth and eighth days from bacterial infection. One patient had no evidence of marrow engraftment and died on the 24th day of infection. The marrow at autopsy was extremely hypocellular.

Thirty-three patients gave evidence of marrow engraftment as indicated by rising peripheral blood counts between the 10th and 21st days after marrow infusion. Examination of marrow aspirates and biopsies confirmed marrow engraftment. Twenty-one of the 33 had donors of opposite sex. Cytogenetic analyses of marrow and peripheral blood cells carried out as early as the 14th day after grafting proved engraftment. Only cells of donor karyotypes have been found up to the present in surviving patients. In other patients, other blood genetic markers such as red-cell antigens and enzymes were used to monitor allogeneic engraftment. After initial marrow engraftment, six patients rejected their grafts, with progressively falling peripheral blood counts leading to marrow hypoplasia. Five of the six died between the 33rd and 67th days with infection. One of the six showed autologous marrow recovery as evidenced by cytogenetic analyses of marrow and peripheral blood cells and is well more than one year later. Of the patients with a graft, one died with a fungal infection on the 18th day, three died from cytomegalovirus pneumonia between the 5th and 91st days and one died of unknown causes on the 427th day. Six patients died with graft-versus-host disease between the 19th and 95th days. Sixteen patients are alive, with functioning grafts.

The results show that normal allogeneic hemopoietic stem cells can repopulate the marrow in patients with aplastic anemia and that the disease is usually related to a failure of the hemopoietic stem cells. An exception to this rule may be the one patient with autochthonous marrow recovery after rejection of the marrow transplant from his sister. Our experience shows that long-term stable chimerism is possible in man. Clearly, major problems remain to be solved. Even so, the survival and hematologic reconstitution of almost half our patients with advanced aplastic anemia treated by allogeneic marrow grafts compare favorably with results obtained by conventional management of this disease. Initially, we restricted this procedure to critically ill patients. The results, however, indicate that marrow transplantation has emerged as a definitive form of therapy for aplastic anemia provided an HL-A matched sibling can be identified as a potential marrow donor. Marrow grafting should be considered before major infection and refractoriness to blood transfusions occur. Other centers have now reported successful marrow grafts in aplastic anemia that confirm this suggestion.

Acute Leukemia

Syngeneic (monosygotic twin) transplants. Since lethal total-body irradiation can exert a definite anti-leukemic effect, and since syngeneic marrow infusion can prevent death from the irradiation-induced aplasia, the rare leukemic patient with advanced disease who is fortunate enough to have a normal identical twin is the logical candidate for attempts to eradicate the leukemia by total-body irradiation and twin marrow transplantation. Very early encouraging results in mice prompted Thomas et al. to try such an approach in three patients with acute lymphoblastic leukemia. Although normal hemopoiesis was restored, leukemia recurred within seven to 12 weeks. A fourth patient with acute lymphoblastic leukemia received cytosine arabinoside before the total-body irradiation and methotrexate after the marrow transplant, but still relapsed within seven weeks (Table 1).

Beginning in 1969 potential "immunotherapy" was added to the basic regimen of total-body irradiation and twin marrow with the hope of delaying leukemic recurrence. It was based on principles outlined in the above section on studies in rodents and on studies supporting the existence of human leukemia-associated antigens. Since we would not expose normal twins to tumor material, the immunotherapy after total-body irradiation and marrow transplantation consisted of infusion of normal-twin buffy-coat lymphocytes three times per week for three weeks and weekly subcutaneous injections of lethally irradiated, autologous, leukemic cells, in the hope of providing a continual antigenic stimulus to the infused donor lymphocytes. The leukemic cells were stored at -180°C in 10 per cent dimethyl sulfoxide and irradiated with 10,000 rad to prevent cell replication. The results obtained in three patients treated with total-body irradiation, marrow transplantation and immunotherapy are presented in Table 1.

Table 1. Treatment of Hematologic Neoplasia by Transplantation of Identical-Twin Marrow

<table>
<thead>
<tr>
<th>THERAPY</th>
<th>NO. OF PATIENTS</th>
<th>NO. IN REMISSION &gt; 1 Mo</th>
<th>DURATION OF REMISSION (Mo)</th>
<th>NO. NOW ALIVE IN REMISSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irradiation</td>
<td>4</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Irradiation +</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>7</td>
<td>5</td>
<td>&gt;3, 4, 4, *6</td>
<td>2</td>
</tr>
<tr>
<td>Cyclophosphamide +</td>
<td>13'</td>
<td>10</td>
<td>3, 4, 5, 7</td>
<td>&gt;8, &gt;16</td>
</tr>
<tr>
<td>Irradiation +</td>
<td></td>
<td></td>
<td>&gt;31, &gt;31</td>
<td>&gt;32, &gt;49</td>
</tr>
</tbody>
</table>

*Death at 4 mo of pneumonia in remission.

*1 patient died of pneumonitis at 1 mo while in remission.
shown in Table 1. A 15-year-old patient with acute lymphoblastic leukemia relapsed within a month, a 26-year-old patient with acute myelogenous leukemia went into remission but died of interstitial pneumonitis at seven weeks, and a 33-year-old patient with acute myelogenous leukemia relapsed at 10 months.

We assumed that the addition of a high dose of chemotherapy to the treatment regimen would be beneficial by decreasing the tumor load to be handled by the total-body irradiation or immunotherapy or both. Therefore, 20 subsequent patients received cyclophosphamide (60 mg per kilogram on each of two days), total-body irradiation (1000 rad), twin marrow transplants and, in 13 cases, immunotherapy. All patients had received chemotherapy and were considered to be in the terminal phase of the illness. The seven patients who did not receive immunotherapy either had not had readily accessible tumor cells for storage or had normal-twin donors who were unable to donate regularly the necessary buffy-coat cells. Thus, this was not a randomized study to determine the contribution, if any, of immunotherapy to the overall results. The results are shown in Table 1. One patient died of hepatitis too early to be evaluated, and two failed to clear their marrow of leukemic cells. Seventeen experienced complete remission. Eight of them (three with acute lymphoblastic, four with acute myelogenous, and one with lymphosarcoma leukemia) remain in complete remission at three to 49 months without any maintenance chemotherapy. All the normal-twin donors remain in excellent health without evidence of hematologic neoplasia.

As predicted from rodent models, although normal hemopoiesis was restored in our twin transplant recipients, the principal problem was that of resistance of the hematologic neoplasms in almost half our patients, as reflected by persistence of leukemic cells or recurrence of leukemia after several months of remission. This resistance has also been a problem in four patients with leukemia treated by others with high-dose combination chemotherapy with marrow infusion from a normal twin—all relapsed within a few months. Thus we are now adding intensive combination chemotherapy before the cyclophosphamide and total-body irradiation to reduce further the tumor burden. Our results are sufficiently encouraging to recommend this approach to any patient who has an identical twin and hematologic neoplasia even before the patient becomes demonstrably resistant to any chemotherapy. Furthermore, this approach may also be applicable to the treatment of other tumors such as lymphomas, neuroblastomas, and anaplastic carcinomas known to be sensitive to high doses of chemotherapy and total-body irradiation.

Allogeneic transplants. The majority of marrow grafts for acute leukemia have been performed between ABO-compatible, HLA-identical siblings and siblings not reactive in mixed leukocyte culture. The main differences in the approach to marrow grafting have centered on methods of conditioning the patient for the marrow graft and of postgrafting immunosuppression. Table 2 describes the approach adopted in several institutions since 1970. Of the conditioning regimens so far tried, reductive, high-level chimerism and sustained remission have been obtained only with cyclophosphamide in doses of at least 45 mg per kilogram per day for four days, high-dose chemotherapy (BACT) and, as will be described, total body irradiation alone or in combination with cyclophosphamide. Conditioning with antilympho-

Table 2. Summary of Recently Reported Results of Marrow Transplantation for Acute Leukemia Using Irradiation, Cyclophosphamide, Antilymphocyte Serum, or Combination Chemotherapy for Conditioning.*

<table>
<thead>
<tr>
<th>Conditioning Regimen</th>
<th>NO. OF PATIENTS</th>
<th>GRAFT &quot;TAKES&quot;</th>
<th>POST-TRANSPLANT IMMUNOSUPPRESSION</th>
<th>NO. SURVIVING IN REMISSION</th>
<th>SURVIVAL (DAYS)</th>
<th>CAUSE OF FAILURE</th>
<th>SOURCE OF DATA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CY I</td>
<td>1</td>
<td>None</td>
<td>0</td>
<td>90</td>
<td></td>
<td>Leukemia</td>
<td>Meuwissen et al 197</td>
</tr>
<tr>
<td>CY II</td>
<td>8</td>
<td>CY+ (8)</td>
<td>0</td>
<td>13-215</td>
<td></td>
<td>GVHD (4), infection (3), leukemia (1)</td>
<td>Santos et al 198</td>
</tr>
<tr>
<td>CY III</td>
<td>1</td>
<td>--</td>
<td>0</td>
<td>77</td>
<td></td>
<td>Infection</td>
<td>Pruzanski et al 199</td>
</tr>
<tr>
<td>CY III</td>
<td>8</td>
<td>CY (1) or MTX (6)</td>
<td>None (2)</td>
<td>10-602</td>
<td></td>
<td>GVHD (1), infection (1), leukemia (6)</td>
<td>Graw et al 200</td>
</tr>
<tr>
<td>950 rad</td>
<td>1</td>
<td>None</td>
<td>0</td>
<td>33</td>
<td></td>
<td>GVHD</td>
<td>Graw et al 200</td>
</tr>
<tr>
<td>ALS</td>
<td>10</td>
<td>MTX</td>
<td>0</td>
<td>60-180</td>
<td></td>
<td>Partial chimerism, no antileukemic effect</td>
<td>Amiel et al 201</td>
</tr>
<tr>
<td>ATO + 735 rad</td>
<td>2</td>
<td>MTX</td>
<td>0</td>
<td>94-122</td>
<td></td>
<td>Leukemia (1), infection (1)</td>
<td>Gregoelian et al 202</td>
</tr>
<tr>
<td>BACT</td>
<td>4</td>
<td>CY (3), MTX (1)</td>
<td>1</td>
<td>3-600</td>
<td></td>
<td>Cardiac toxicity (1), GVHD (1), infection (1)</td>
<td>Graw et al 203</td>
</tr>
</tbody>
</table>

*Figures in parentheses represent no. of patients. CY denotes cyclophosphamide, ALS antilymphocyte serum, ATO vincristine, doxorubicin, vinblastine, aclacinomycin, doxorubicine, ester of cyclophosphamide, ATO vincristine, doxorubicin, vinblastine, aclacinomycin, doxorubicine, ester of cyclophosphamide, GVHD graft-vs-host disease.

- CY: Donor autologous (U whole blood) on day -3; CY 37.5 mg/kg on days -2 & -1.
- CY II: Donor autologous (U whole blood) on day -5; CY 50-60 mg/kg on days -4, -3, -2 & -1.
- CY III: Donor autologous on day -5; CY 45 mg/kg on days -4, -3, -2 & -1.
- ALS: 10-20 ml of bone marrow on days 5-12 days before marrow infusion.
- BACT: CY 45 mg/kg on days -5-6; TCH 10 mg/kg body weight on days 1, 3, 6, 11 & then every week.
- ATO + 735 rad: TCH 10 mg/kg on days 1, 3, 6, 11 & then every week.

*HLA = A identical siblings.

**CY: 5-7.5 mg/kg on days 6, 8, 10, and 12 i.v post-transplant. ± oral CY for 3 mo.
- MTX: 10 mg/m² on days 1, 3, 6, 11 & then every week.

'were not HLA identical.
cyte serum alone has not demonstrated an antileukemic effect or high-level chimerism.

In Seattle, 1000-rad total-body irradiation, initially alone and subsequently with the addition of cyclophosphamide and, more recently, cyto reduction as described, have been used as the conditioning regimens in the 70 patients receiving marrow grafts for acute lymphoblastic or acute myelogenous leukemia between July, 1969, and June, 1974 (Table 3). A review of this five-year experience illustrates the changing problems but the definite progress evident in marrow grafting for acute leukemia.

Table 3. Allogeneic Marrow Transplants for Acute Lymphoblastic (ALL) and Acute Myelogenous (AML) Leukemia Using HLA-Matched Sibling Donors (Seattle Experience).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TOTAL PATIENTS</th>
<th>NO. IN REMISSION &gt;3 M</th>
<th>RECURRENT LEUKAEMIA</th>
<th>SURVIVAL OF PATIENTS NOW IN REMISSION (Mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NO.</td>
<td>MEDIAN RANGE TIME TO RELAPSE (Mo)</td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>34</td>
<td>19</td>
<td>2.5 0-18</td>
<td>49, 31, 20, 19, 12, 5, 4, 3</td>
</tr>
<tr>
<td>AML</td>
<td>36</td>
<td>14</td>
<td>1.5 0-6</td>
<td>29, 23, 16, 12, 7, 5, 4, 3</td>
</tr>
<tr>
<td>CMU</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

*Of these patients were prepared with total-body irradiation only.
1 Patients relapsed with central nervous system leukemia at 18 mo; all other patients relapsed in <7 mo.
2 Chronic myelogenous leukemia in blast crisis.

Thirty-six patients, two to 56 years of age (average of 23 years) received marrow grafts for acute myelogenous leukemia, and 34 patients, five to 22 years of age (average of 11 years) for acute lymphoblastic leukemia. Relevant to evaluating this approach is the clinical condition of these patients at the time of grafting. All patients received transplants at a time when conventional and experimental chemotherapy was failing. The duration of disease from diagnosis averaged 24 months in patients with acute lymphoblastic leukemia, spanning an average of three relapses, and in acute myelogenous leukemia, it averaged 10 months, with 15 patients not achieving a remission with chemotherapy. Overall, 64 of the 70 patients were in relapse, 68 had previously been transfused with red cells or platelets (or both), and 22 were infected at the time of transplantation.

Two patients died in less than 10 days, and five did not show engraftment. Sixty-three obtained functional grafts demonstrated by rapidly rising peripheral counts, histologic evidence of a graft, and, when possible, cytogenetic analysis and red-cell or white-cell antigen or enzyme studies. In 46 of these 63 patients (73 per cent) graft-versus-host disease developed, ranging from an erythematous skin rash lasting only several days to the fatal syndrome of a desquamating erythematous skin rash, gross liver-function abnormalities and profuse diarrhea. There was no difference in the incidence of graft-versus-host disease in the two disorders — 21 of 31 patients with acute lymphoblastic and 25 of 32 patients with acute myelogenous leukemia.

The overall survival data for this group of patients are shown in Figure 2. Although the median survival for patients with the two types of leukemia remains short, the five-year period is characterized by marked improvement in supportive care of patients and more effective conditioning regimens. Analysis of the results according to the year of transplantation demonstrates the progress now evident in two respects: the prolongation of survival beyond the first 50 days; and the increasing number of long-term survivors. Thus, the percentage of patients surviving beyond 50 days has increased from less than 50 per cent in those receiving transplants between 1969 and 1971 to 100 per cent in those with transplants in the first six months of 1974.

Relapse was the basic cause of failure in 15 patients, four with acute myelogenous and 11 with acute lymphoblastic leukemia. Included are four patients (two in each group) whose leukemic cell population was so refractory that serial marrow aspirates demonstrated persistence of blast cells. Such refractoriness suggests a spectrum of degrees of reduction of the leukemic cell load. One could anticipate, therefore, that a substantial fraction of the patients might retain a malignant cell population large enough for a putative antileukemic effect of a marrow graft to do much good. In fact, we have observed four patients in whom leukemia recurred in seven to 23 weeks after marrow engraftment, and in whom cytogenetic evidence showed that the recurrence was composed of cells derived from the original host leukemia. Furthermore, the more recent aggressive conditioning regimens appear to have modified the relapse rate. Of the first six patients with acute lymphoblastic leukemia prepared with total-body irradiation alone, five had evidence of recurrence (including a child who died primarily of bilateral interstitial pneumonia and had extramedullary microscopic evidence of leukemia). With the subsequent addition of high-dose chemotherapy to the conditioning regimen, only six of 29 patients with acute lymphoblastic leukemia have shown a recurrence. As the median survival increases, however, more cases of recurrence may also become evident, although relapse beyond seven months has occurred in only one patient.

Nineteen of the 70 patients remain in complete remission beyond three months, nine for one to four years after transplantation. From the data presented, indications are that such a remission rate is increasing (Fig. 2). In many patients with acute myelogenous leukemia and adult acute lymphoblastic leukemia, and after relapse in acute leukemia of any kind, long-term unmaintained remissions are not possible with available chemotherapy. The results with marrow transplantation, far from ideal, are nonetheless encouraging and offer a therapeutic opportunity for the patient with a suitable donor whose leukemia by virtue of type or relapse augurs a predictably short prognosis.

The results of marrow grafting for the blastic crisis of
chronic myelogenous leukemia cannot yet be evaluated. Two patients died early of infection, and one with a graft is in remission after four months (Table 3).

Current Results in Miscellaneous Conditions

Three other applications of marrow grafting have long been recognized: radiation accidents; solid malignant tumors that are particularly sensitive to chemotherapy and radiotherapy; and genetic disorders such as thalassemia and sickle-cell disease.

Apart from transplants after radiation accidents, there are no published reports of long-term survival after allogeneic transplants for the disease categories listed. In these disorders either the transplantations have been performed before the recognition of the importance of HLA and mixed-leukocyte-culture matching, patients having succumbed to overwhelming infections in the early post-grafting period, or there has been no evidence of engraftment.

Within our center allogeneic marrow grafting has been carried out in single patients with neuroblastoma, malignant histiocytosis, and Hodgkin’s disease. All succumbed within 40 days of transplantation, two from overwhelming sepsis and one from viral pneumonia.

A major factor limiting the total eradication of a disseminated malignant neoplasm by current available chemotherapy and radiotherapy is irreversible damage to the marrow. Transplantation of marrow should remove this barrier and would permit the administration of much larger doses of these agents. Neuroblastoma, lymphosarcoma, Hodgkin’s disease and oat-cell carcinoma are examples of tumors that appear to be particularly sensitive to chemotherapy and to radiotherapy, and in such neoplasms the application of marrow transplantation may enable potentially curable doses to be given.

Two approaches are feasible: the infusion of the patient’s own normal marrow aspirated and stored before chemotherapy and radiotherapy (autologous marrow infusion); or the use of marrow from a major-histocompatibility-complex-matched donor. In the late 1950’s there were several case reports of remissions with use of autologous marrow infusion after high doses of chemotherapy or radiotherapy or both. Death of most patients from bacterial sepsis and the doubt whether marrow recovery in the long-term survivors was in any way related to the marrow infusion led to a general loss of interest in this technic. The development of methods for long-term storage of marrow at -180°C in dimethyl sulfoxide, better chemotherapy agents, and more effective supportive care of patients have renewed interest in this form of therapy, which, like identical twin transplants, does not carry the risk of rejection or graft-versus-host disease. Currently, we are storing marrow from patients with chronic myelogenous leukemia early in their disease. When blast crisis occurs, the patient will be treated with intensive chemotherapy and radiotherapy followed by return of the stored marrow, with the intent of restoring the chronic disease. Preliminary results indicate feasibility.

Current Problems

Success or Failure of Marrow Engraftment and Marrow-Cell Rejection

A review of the literature of human marrow transplantation before 1967 showed a high incidence of complete failure of engraftment. Failure of initial allogeneic engraftment is no longer a major problem. Excluding five patients who died too soon to be evaluated, 33 of 34 grafts
in patients with aplastic anemia were successfully established, and 63 of 68 in acute leukemia were successful. The quantity of marrow infused ranged from 1.1 to 10.9 x 10^8 per kilogram of recipient body weight (Table 4). Despite this range of one order of magnitude, there was no correlation between marrow dose and success or failure of engraftment nor of nadir of white cell count or time to recovery above 1000 per cubic millimeter. Although there has been concern about the dose of marrow cells required for engraftment, 116 precise determination of this number seems to be neither practical nor necessary.

Experiments in canine littermates matched at the major histocompatibility complex have shown that prior exposure to transudation of whole blood from the marrow donor may jeopardize the success of a subsequent marrow graft even in this "compatible" donor-recipient combination. 50, 51 Data similar to those in dogs have been reported in irradiated mice 115 and also in mice treated with cyclophosphamide. 19 Presumably, rejection was due to immunization of the recipient to histocompatibility antigens on platelets and leukocytes. With blood transusions from random donors or from family members other than the marrow donor, immunization of the recipient and rejection of the graft might be expected only when the marrow donor and the transfusion donors share "minor" histocompatibility antigens not present in the recipient. The results of allogeneic marrow grafting for the treatment of aplastic anemia have shown that patients given transfusions from family members have a high rate of failure of marrow engraftment or of marrow-graft rejection. 100 The two patients who had not been transfused before grafting had prompt engraftment and are among the long-term survivors. One patient who rejected his graft had had transfusions from both parents, which should have exposed him to the risk of sensitization to all family "minor" transplantation antigens that he did not inherit. Although Mathé et al. 15 had earlier reported an apparent harmful effect of transfusions before grafting in patients with leukemia, we have not observed graft rejection in our patients with acute leukemia, and most of the failures of engraftment occurred in the earlier patients. More aggressive modern chemotherapy is one explanation for this difference as well as the difference between acute leukemia and aplastic anemia.

At present no reliable laboratory tests are available to determine which patient is sensitized against his HLA-

Table 4. Marrow Cells Infused and Days Required for White-Cell Count (WBC) to Go Above 1000 Per Cubic Millimeter.

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>NO. ANALYZED</th>
<th>MARROW CELLS INFUSED</th>
<th>DAY WBC &gt; 1000/CUBIC MILLIMETER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEDIAN RANGE</td>
<td>RANGE</td>
<td>MEDIAN RANGE</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>34</td>
<td>2.6</td>
<td>1.1-9.4</td>
</tr>
<tr>
<td>Acute lymphoblastic</td>
<td>31</td>
<td>3.3</td>
<td>1.4-16.9</td>
</tr>
<tr>
<td>leukemia</td>
<td>32</td>
<td>3.0</td>
<td>1.1-6.4</td>
</tr>
</tbody>
</table>

matched marrow donor although research on this problem is in progress. 116, 117 Recent data using DLA-incompatible, unrelated donor recipient pairs of dogs indicate that the present state can be abrogated by a combination of procarbazine and rabbit antidog antithymocyte serum before total-body irradiation resulting in successful marrow engraftment in most cases. 95 We have initiated a randomized study in patients with aplastic anemia to determine the role of pretreatment with procarbazine and antithymocyte globulin. Initial results have shown that the regimen is well tolerated and that successful engraftment is possible.

**Graft-versus-Host Disease**

The distinction between illness due to the active immunologic assault of donor lymphoid cells against host target organs and the consequences of this assault, delayed organ function and infection, is indeed subtle and, in this discussion, both are considered a part of graft-versus-host disease. For unknown reasons, the principal target organs of graft-versus-host disease in both animals and man are skin, gastrointestinal tract and liver. 11, 28 Despite the use of sibling donors matched at the major histocompatibility complex and despite post-grafting immunosuppression, graft-versus-host disease has occurred in approximately 70 per cent of patients with successful marrow grafts. Evidently, compatibility or incompatibility for histocompatibility regions other than the major histocompatibility complex are important determinants of the graft-versus-host disease. There have been too few successful marrow transplantations between siblings not matched at the major histocompatibility complex to know the frequency of graft-versus-host disease in this situation, but, from studies in animals as well as from the results of marrow grafting in immunodeficient children, 49 it seems reasonable to expect that graft-versus-host disease (and graft rejection) will be major problems. We described a marrow transplant involving a one-haplotype mismatch. 118 Graft-versus-host disease was severe but not more severe than that seen in some transplants matched at the major histocompatibility complex.

**Clinical graft-versus-host disease.** The proposed clinical staging of the disease is shown in Table 5. The initial organ

Table 5. Proposed Clinical Stage of Graft-versus-Host Disease According to Organ System.

<table>
<thead>
<tr>
<th>STAGE</th>
<th>SKIN</th>
<th>LIVER</th>
<th>INTESTINAL TRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Maculopapular rash &lt;25% of body surface</td>
<td>Bilirubin 2-3 mg/100 ml</td>
<td>&gt;500 ml diarrhea/day</td>
</tr>
<tr>
<td>++</td>
<td>Maculopapular rash 25-50% body surface</td>
<td>Bilirubin 3-6 mg/100 ml</td>
<td>&gt;1000 ml diarrhea/day</td>
</tr>
<tr>
<td>+++</td>
<td>Generalized erythroderma</td>
<td>Bilirubin 6-15 mg/100 ml</td>
<td>&gt;1500 ml diarrhea/day</td>
</tr>
<tr>
<td>++++</td>
<td>Generalized erythroderma with bullous formation &amp; desquamation</td>
<td>Bilirubin &gt;15 mg/100 ml</td>
<td>Severe abdominal pain, with or without Reus</td>
</tr>
</tbody>
</table>
involved in almost all cases is the skin. Biopsy is mandatory for diagnosis since not all rashes in these patients are due to graft-versus-host disease. Hepatic and intestinal involvement usually appears several days after the rash. Intestinal involvement is mainly in the form of diarrhea but may progress to abdominal pain and ileus. Liver disease is manifested by rises in bilirubin (mainly conjugated). Serum glutamic oxaloacetate transaminase is usually in the range of 150 to 750 IU. Rises in alkaline phosphatase are also observed especially in chronic graft-versus-host disease. Intestinal and hepatic abnormalities are not unique to this situation. Therefore, alterations in function of these organs should be attributed to graft-versus-host disease only when its involvement of the skin is clearly established or when there is biopsy confirmation in gut or liver. Fever, wasting and a decreased performance status are also regularly seen with severe involvement. Although fever may be a manifestation of graft-versus-host disease per se, it is most commonly due to an associated infection, often an occult viral infection.

We and others have attempted to classify the overall severity of clinical graft-versus-host disease on a grading system ranging from 0 to 1V (Table 6). Currently, analysis of survival among our patients suggests that patients can be grouped in two ways: those without clinically evident graft-versus-host disease (Grade 0) and those with graft-versus-host disease involving skin (Grade 1) show a survival of 55 per cent, whereas patients with Grades II to IV have a survival of only 15 per cent. Its strong association with infection indicates that graft-versus-host disease itself is a critical factor contributing to the poor immunologic reactivity, debility and compromised mucosal barriers in narrow-graft recipients. The grading system may become more meaningful as advances are made in the treatment of graft-versus-host disease and accompanying infections.

Chronic graft-versus-host disease characterized by a more indolent clinical course, with involvement of skin, liver and intestinal tract, has been observed in three long-term survivors. This picture may appear after complete or partial resolution of acute graft-versus-host disease. Whether the chronic form is the result of an immunologic attack of donor lymphoid cells against host tissue or of deficient immunologic reactivity of the graft, with accompanying opportunistic infections, or of a combination of both is not well understood at present.

Table 6. Overall Clinical Grading of Severity of Graft-versus-Host Disease.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Degree of Organ Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+ +  + skin rash; no gut involvement; no liver involvement; no decrease in clinical performance.</td>
</tr>
<tr>
<td>II</td>
<td>+ +  + skin rash; + gut involvement or + liver involvement (or both); mild decrease in clinical performance.</td>
</tr>
<tr>
<td>III</td>
<td>+ +  + + skin rash; + + gut involvement or + + liver involvement (or both); marked decrease in clinical performance.</td>
</tr>
<tr>
<td>IV</td>
<td>Similar to grade III with + +  + + organ involvement &amp; extreme decrease in clinical performance.</td>
</tr>
</tbody>
</table>

Histologic criteria. Table 7 summarizes the proposed histopathologic staging of graft-versus-host disease based on scale ranging from + to + + + +. Hepatic involvement was characterized by degeneration and eosinophilic necrosis of parenchymal cells and degeneration and necrosis of the epithelium of small bile ducts. Because the effect on parenchymal cells was variable whereas small bile ducts were consistently involved, the grading was correlated with the percentage of pathologically altered small bile ducts.

Serial biopsies of the skin are easily obtained, but biopsies of the gut and liver cannot be done regularly and the histopathologic stage may therefore require revision. Histopathologic staging varied between organs and did not always correlate with the clinical grade except for mild or severe graft-versus-host disease.

Prevention and treatment. From the animal studies as well as from the clinical narrow-grafting experience it has become clear that some form of immunosuppressive therapy must be used after grafting to diminish or prevent graft-versus-host disease. The literature on this topic has recently been reviewed. To be effective, treatment with immunosuppressive agents must be started before graft-versus-host disease has become apparent. Of the many agents studied, methotrexate and cyclophosphamide have been found to be useful. Methotrexate was found to ameliorate graft-versus-host disease in mice, dogs and monkeys when given immediately after grafting. Studies in dogs have shown that methotrexate was most effective when continued for a long time (three months), and stable long-term chimerism (measured in years) was achieved in some DL-A incompatible recipients. Cyclophosphamide had some effect in mice, rats and monkeys, but was ineffective in dogs. Studies using cytosine arabinoside, procarbazine, 6-mercaptopurine, and antilymphocyte serum in the immediate post-grafting period have been disappointing.
Prevention of graft-versus-host disease has been attempted with use of antilymphocyte serum given just before grafting in rodents, dogs, and monkeys, with only slight prolongation of survival.

Our current post-grafting regimen in human patients consists of methotrexate, 15 mg per square meter on the first day and 10 mg per square meter on the third, sixth and 11th days and weekly thereafter for the first 100 days. Santos et al. have used cyclophosphamide, 7.5 mg per kilogram for five doses on alternate days, beginning on the first day after marrow grafting, followed by doses at irregular intervals. The frequency of fatal graft-versus-host disease in patients given a marrow graft from an HL-A-matched sibling and no post-grafting immunosuppression is unknown. Despite post-grafting immunosuppression with either methotrexate or cyclophosphamide, severe and fatal graft-versus-host disease has been observed in 10 to 20 per cent of human marrow-graft recipients.

Surprisingly enough, only a few studies have been reported in which established graft-versus-host disease was treated either with cytostatic agents or with antilymphocyte serum. For the most part, these studies were negative—i.e., the agents used failed to influence the course of events or the authors failed to document sustained chimerism. A review of these studies has been presented recently. An exception was the study by Owens and Santos, who inoculated (C57BL/6 X DBA 2) F1, hybrid mice with BALB/c spleen cells and were able to suppress clinically established graft-versus-host disease by cyclophosphamide. Cyclophosphamide was used to treat established graft-versus-host disease in a patient given a graft from an HL-A-matched sibling and was ineffective.

On the basis of encouraging studies in the canine model we have recently started using antilymphocyte globulin for treatment of human patients with graft-versus-host disease. With this therapy, 12 of 19 patients showed complete resolution of the graft-versus-host disease, live showed improvement of most organ systems involved, and two showed no changes except for improvement in skin lesions. Six of the 19 became long-term survivors. Five of the six are alive now between 525 and 865 days after grafting, and one died on the 346th day with chronic respiratory failure. Of the remaining 13 patients, 11 died of interstitial pneumonitis of predominantly viral origin, and two died with fungal and bacterial infections. These findings demonstrated the partial effectiveness of antilymphocyte globulin in reversing human graft-versus-host disease. This treatment of established graft-versus-host disease certainly does not present the solution of the problem, but it appears to have modified the grim outlook in a hopeful way. It is obvious, however, that continued animal and clinical research efforts have to be directed to a better understanding of the nature and control of graft-versus-host disease.

Albumin-gradient cell-separation techniques have been used in an attempt to eliminate immunocompetent cells from the marrow inoculum while retaining the hemopoietic stem cell. Proof, however, that this approach is useful in modifying graft-versus-host disease in grafts between random bred non-rodent animals is missing. The technic has been applied in a number of human transplantations. The result was either failure of engraftment or death from graft-versus-host disease when histoincompatible marrow was used. Aside from the technical problems, a number of theoretical reasons would argue against the efficacy of "stem-cell" separation technics. Even if it should be possible to separate completely lymphoid cells from stem cells, it is likely that the recipient still might be susceptible to the late form of graft-versus-host disease, which is presumably caused by a subsequent regeneration of reactive lymphoid cells derived from the "common" stem cell. If there is not a "common" stem cell giving rise to lymphoid cells, successful separation would create a recipient who is severely deficient in immune function and thus might succumb to infection. At present, therefore, stem-cell separation does not appear to be a promising approach.

Infection after Engraftment

Leukopenia, immunoincompetence and graft-versus-host disease have, as their principal consequence, infection, which is the usual proximate cause of death. These complications may overlap in time and clinical manifestation, but it is possible to discern time periods in which one or the other underlying cause predominates.

The infectious hazards of agranulocytosis are maximally apparent during the two to three weeks immediately after transplantation. In a series of 71 allogeneic marrow-transplant recipients who survived for at least 30 days, and who achieved engraftment, the median number of days with a granulocyte count of < 100 per cubic millimeter was 11, and that of patients with a count of < 500 was 18. The pattern of infection in agranulocytic patients has been well delineated, mostly in patients with acute leukemia, and infectious-disease experience in marrow-transplant patients during the immediate post-grafting period conforms closely to this pattern. Bacterial and candidal infections are particularly frequent and serious in agranulocytic patients. Gram-negative bacteremia is a common event. There is convincing evidence that antibiotic therapy is less effective in curing infectious disease in patients lacking granulocytes and that the apparent complete resolution of an infectious process may be insecure unless adequate granulocytes are present. Organisms usually considered of low virulence acquire enhanced consequence in the absence of granulocytes. The frequency of systemic candidiasis is difficult to assess since technics for its detection are of low sensitivity. Mucosal infections due to candida, however, are common. Bacterial infection appropriately treated rarely jeopardizes the achievement of successful engraftment, even in patients infected at the time the facilitation procedure is initiated, but it does cause considerable morbidity. Of 47 patients surveyed in a recent review, only two died from bacterial disease in the first 21 days, but in 22 bacteremia developed. Aside from the role of granulocyte transfusion the recently improved survival prospects of infected agranulocytic patients owe much to the development of new and more po-
tent antibiotics properly administered.

Because of decreased immune reactivity in these patients, it is not surprising that the incidence of infection, particularly viral disease, is increased during the first 100 days after transplantation. Perhaps the most troublesome single infectious complication is that of interstitial pneumonia. The syndrome has occurred at any time during the first four months after transplantation, but has its usual onset after the establishment of a functioning marrow graft. The overall incidence among 114 marrow-graft recipients in Seattle over the time period from 1969, through June, 1973, is 28 per cent. However, in the 24 identical-twin transplants in this series, the condition was documented in only three patients (13 per cent). Furthermore, as previously discussed, some allogeneic marrow recipients died without achieving effective hematopoietic function (or after marrow-graft rejection) in the period before the risk of interstitial pneumonia is very great. If these are excluded, and we consider only patients who achieved functional allogeneic transplants, the incidence of this syndrome is closer to 50 per cent. Mortality among this group of patients, death being primarily due to respiratory insufficiency from progressive pulmonary infiltration, is distressingly high at between 50 to 60 per cent. Whereas all groups of allogeneic marrow-graft recipients have contracted this syndrome, both the statistics on incidence and particularly those on mortality are worse in patients with grades II to IV graft-versus-host disease. In this connection, an apparent increase in incidence of interstitial pneumonia in marrow-transplant recipients has been reported in Seattle. However, it remains to be established whether this observation reflects a genuine increase in the attack rate or a manifestation of the enhanced chronicity of severe and moderate graft-versus-host disease (producing a longer period of maximum risk) resulting from the introduction of new methods of support and immunosuppressive treatment discussed above.

From the standpoint of cause about half the patients dying with interstitial pneumonia have unambiguous evidence of disseminated cytomegalovirus in the form of characteristic inclusion bodies on histologic examination of the lungs and other organs, as well as isolation of the organism in culture. A total of six histologically proved cases of Pneumocystis carinii has been noted at autopsy, three of which were found in the presence of cytomegalovirus. In two patients lethal varicella-zoster pneumonia developed. No recognizable pathogens have been associated with the remainder of the fatal interstitial pneumonias, although these are histologically indistinguishable from those caused by cytomegalovirus and pneumocystis (except for the specific signs of these agents). Of marrow-graft recipients who gave clinical evidence of interstitial pneumonia and subsequently recovered, about half also had serologic or cultural (or both) evidence of cytomegalovirus infection. The cause in the other half remains obscure.

A retrospective review of cytomegalovirus infection and serologic responses among marrow-graft recipients provided the following observations: cultural and histologic data taken together indicated that a minimum of one third of these patients contract or activate latent cytomegalovirus infections during the first few months after engraftment; and patients with marked rises in complement-fixing antibody titer either had no clinical manifestations or recovered from transient interstitial pneumonia. Most of these patients excreted cytomegalovirus in urine. In contrast, patients with fatal cytomegalovirus pneumonia rarely demonstrated a noteworthy rise in complement-fixing antibody titer and have not, in our experience, excreted cytomegalovirus in urine at the time of the disseminated infection. The implications of these findings, as well as their precise relation to immune reconstitution and other factors that might affect the pattern of response to cytomegalovirus infection in transplant recipients, deserve more thorough prospective study. Finally, very little can be said about the clinical management of interstitial pneumonia and disseminated cytomegalovirus infection. All the severely infected patients have received antibiotics and pentamidine isethionate. Cytosine arabinoside, adenine arabinoside and corticosteroids have also been used but there is no clear evidence of benefit from any of these drugs at present.

In contrast to the problems associated with cytomegalovirus infection (and whatever additional and unknown agent or agents may account for interstitial pneumonia) the experience in Seattle with other opportunistic viral infections is relatively modest. Herpes simplex virus infections of the mouth and lips are frequent complications in the first few weeks after transplantation, but, with one exception, have subsided and healed without widespread dissemination. Later, cutaneous herpes zoster occurs occasionally over the period in which cytomegalovirus infections are seen. These lesions have frequently spread over several dermatomes before subsiding. Antibody responses to these viruses have been observed. The documented incidence of P. carinii pneumonia has been referred to and, perhaps surprisingly, is quite low. Antibody responses to this organism are very rare in our population of marrow-graft recipients, with only two documented cases, both of which were not in patients with proved P. carinii pneumonia.

**Immunologic Status of Long-Term Survivors**

**Immunologic reactivity of syngeneic marrow-transplant recipients (monozygotic twins).** Three patients treated with twin marrow grafts for hematologic neoplasia have been tested for immunologic reactivity. Absolute lymphocyte counts were above 1000 per cubic millimeter in all patients within one to three months after transplantation. No consistent changes in serum immunoglobulin levels were noted. Two of these patients — both of whom had received twin peripheral blood lymphocytes as well as marrow — exhibited normal primary and secondary antibody responses to bacteriophage 0x174 at six weeks and at one year after marrow transplantation. Two patients did not exhibit cutaneous delayed hypersensitivity reactions to the usual battery of skin tests, even though one of the donors was positive to candida and mumps. The third patient became positive to candida at one year. Two patients responded to dimethylchlorobenzene at one and two years. Lymphocytes from all three were stimulated in the test...
with mixed leukocyte culture by irradiated allogeneic cells and by phytohemagglutinin to an extent almost comparable to that observed with the normal twin's lymphocytes during the first few months after marrow transplantation. The three long-term syngeneic transplant survivors studied had no serious infections. Thus, the results suggest an impressive restoration of immunologic function in the three patients. It is assumed, of course, that the immunologic system tested is totally of donor type, although no markers are available. Efforts are under way to study the immunologic reactivity of twin marrow-transplant recipients early after marrow transplantation with additional measurements of immunologic function.

Immunologic reactivity of allogeneic marrow-transplant recipients (major-histocompatibility-complex-matched donors). The recovery of immune reactivity in allogeneic chimeras would be expected to be slower than that of syngeneic chimeras owing to at least three possible mechanisms: it has been hypothesized that lymphoid depletion occurs in allogeneic chimeras as a result of destruction of donor lymphoid cells during graft-versus-host disease; allogeneic chimeras have often been treated with immunosuppressive drugs after grafting, which not only delay or prevent graft-versus-host disease but also suppress the response of chimeric lymphocytes to other antigens; and the combination of toxic conditioning regimens, post-grafting immunosuppression and graft-versus-host disease causes gut damage leading to a malabsorption syndrome, which given enough time, can lead to immunodeficiency.128 In general, allogeneic rodent chimeras are less immunologically active than their syngeneic counterparts even if studied as late as 300 days after grafting (reviewed elsewhere).128,129 In a recent study, the immune system of random-bred canine chimeras was surveyed for periods up to eight years after grafting.127 After a prolonged period of decreased immune reactivity lasting for 200 to 300 days after grafting, cellular and humoral immunity in these chimera returned to the normal range. Although there was a delayed rise in antibody titers during the primary immune response, long-term chimera showed normal peak titers and a normal qualitative and quantitative secondary antibody response. Animals were able to live in an unprotected environment without apparent increased occurrence of infection, supporting the conclusion that, after an initial phase of impaired immune function, these canine chimera regained normal immune reactivity.

In an initial study we described evaluation of immunologic reactivity in 10 human recipients of allogeneic marrow observed for five to 20 months after graftment.130 Five patients had hematologic neoplasia, and five aplastic anemia. Serum IgG, IgA and IgM levels declined in all recipients and then returned to normal by the 100th day. Absolute lymphocyte counts were above 1000 per cubic millimeter in all patients within one to three months after grafting. The recipients showed a markedly decreased antibody response to bacteria culture OX174. In contrast, several of these recipients acquired cytomegalovirus or herpes zoster infections from which they recovered, and they developed good antibody titers against these agents. Testing in mixed leukocyte culture showed that recipients of allogeneic grafts had a wide range of responsiveness, but clear stimulation was observed on nearly all occasions even within the first month of grafting. Despite this in vitro activity, the allogeneic recipients, with one exception, could not be sensitized to dinitrochlorobenzene when tested between 28 and 305 days after grafting. We concluded that recipients of allogeneic grafts showed a long-lasting immunologic deficiency that points out the necessity for vigilance in early detection and treatment of infection and the need for additional measures to restore immunologic competence in these patients.

Malignant Transformation of Donor Cells

A mechanism of leukemic relapse that was not anticipated was the possibility of malignant transformation of the engrafted marrow. Nevertheless, there is unambiguous evidence that this event has occurred twice in leukemic marrow-graft recipients. Two female patients with childhood acute lymphoblastic leukemia received total-body irradiation as their only cytoreduction in preparation for engraftment, followed by allogeneic marrow infusion from histocompatible male siblings. Both escaped the serious complications that follow marrow transplantation and appeared to achieve normal marrow status, only to suffer a florid leukemic relapse 69 and 135 days after engraftment. In both cases careful cytogenetic study of a large number of metaphase chromosomes, and in the second case, fluorescent staining for y chromatin in interphase nuclei, indicated that the recurrent leukemia was composed of male cells and was therefore of donor origin.128,129 There are only four other patients in our series who are strictly comparable to these two—that is, children with acute lymphoblastic leukemia prepared only with total-body irradiation. Three of these have also shown leukemic relapse, but in no case was an opportunity presented to determine the host or donor origin of the recurrent leukemia. The one leukemia-free patient in this group is our longest survivor at four years after grafting.

What caused the malignant transformation of the marrow graft? Several explanations have been discussed. It has been suggested that leukemia, at least in some cases, could be a disease of "regulation" such that any marrow in a leukemic host will become leukemic. Another theory advanced involves a cell-fusion event between donor and host cells followed by a loss of extra chromosomal material or "diploidization" with retention of the y chromosome. Still another suggestion is that immunodepression in the marrow-graft recipients could conceivably lead to a breakdown in the putative immune surveillance defenses against spontaneously arising malignant cell clones in the regenerating engrafted tissue. Furthermore, it should be noted that graft-versus-host reactions are themselves leukemogenic, at least in mice.130,131 Against the last two proposals is our failure to observe malignant transformation of marrow grafts in non-leukemic recipients. In addition, leukemogenesis mediated by graft-versus-host disease in mice appears to involve activation of endogenous C-type murine leukemia viruses.132 This point raises a final and perhaps most plausible hypothesis—specifically, that a leukemogenic virus or similar etiologic agent
resident in a recipient infected and transformed the transplanted marrow. Ionizing radiation will induce production of occult C-type leukemia viruses in some experimental animal systems. Moreover, oncovirus infections can be transmitted by fragments of cellular DNA containing an integrated DNA provirus, at least in some systems. Thus, one need not postulate the presence of recognizable whole-virus particles, but rather could envision transmission through the agency of subviral or other oncogenic subcellular material released from the degenerating host leukemic-cell population.

This list may well not exhaust all the possibilities underlying marrow-graft transformation, but it is in the absence of information on the ultimate mechanism, there are a number of questions of immediate consequence. The most important of these is the frequency and prevalence of leukemic transformation. The existence of a mechanism for the frequent induction of neoplasia in new populations of cells in the course of acute leukemia and related diseases might potentially threaten the ultimate success of intensive intermittent-therapy programs dependent upon the stepwise destruction of the tumor-cell population. At present, however, there is no conclusive evidence that induction of malignant transformation in new clones of cells does occur with noteworthy frequency outside of the two cases discussed here and the observation of apparent reintroduction of new tumor clones in certain late relapses of African Burkitt's lymphoma.

Adoptive Immunotherapy

The data on allogeneic marrow transplantation for hematologic neoplasia permit no conclusion about the existence or absence of an antileukemic effect of graft-versus-host disease as anticipated from animal studies. The principal evidence that graft-versus-host disease, whether or not clinically detectable, did exert an antileukemic effect stems from the observation that leukemia recurred more frequently in recipients of twin marrow than in recipients of allogeneic marrow — in which the potential for graft-versus-host disease existed. Analysis of data on the patients whose leukemia cleared from the marrow and who survived for more than 60 days reveals that 13 out of 22 such patients exhibited recurrent leukemia after twin marrow grafts whereas leukemia recurred in only 15 out of 49 patients treated with an allogeneic graft. It must be emphasized, however, that clinically detectable graft-versus-host disease does not correlate with presence or absence of recurrence of leukemia. Most importantly, clinically detectable graft-versus-host disease is not essential for long-term, complete remissions. Of nine long-term survivors free of acute lymphoblastic leukemia, seven never had clinically evident graft-versus-host disease, and neither did five of nine patients whose leukemia relapsed. Of the long-term survivors free of acute myelogenous leukemia six of 10 did have graft-versus-host disease, but so did two patients whose leukemia recurred. Thus, it is not possible to recognize an antileukemic effect of graft-versus-host disease from the data currently available.

Nature of "Tolerance"

In a stable long-term chimera, the immunologically competent foreign graft does not mount a harmful reaction against its host. The most common explanation is that the lymphoid cell clones that would have been reactive against host alloantigens have been made "tolerant" — i.e., specifically non-reactive. Another possibility is that the reactive cell clones have been eliminated in the course of an immunologic interaction. Alternatively, a form of immunologic enhancement may be involved, by which a factor in the chimeric individual's serum can protect against cellular immune reactions. We studied nine canine irradiation chimeras between 173 days and 7.5 years after transplantation of allogeneic marrow. The lymphocyte populations were foreign to their hosts and, therefore, theoretically capable of mounting a graft-versus-host reaction. Lymphocytes from the chimeric dogs were found to inhibit colony formation by their "own" fibroblasts, whereas lymphocytes from other chimeras or from normal dogs did not (the test used was the colony-inhibition assay). Serum from the chimeras specifically abrogated this inhibitory effect. These initial results suggested that the immunologic "tolerance" of the chimeric dog was mediated in vivo by blocking serum substances. Subsequently, similar findings were reported in rodent radiation chimeras, and in mice made tolerant by neonatal injections of allogeneic lymphocytes and in tetraparental mice.

More recently we studied nine canine irradiation chimeras and their DL-A-matched marrow donors between 545 and 1226 days after marrow grafting, using a microcytotoxicity or cell-inhibition assay. Before testing, marrow donors were immunized against their chimeras by repeated skin grafts, which they rejected. Skin fibroblasts from chimeras and their donors were tested for cell inhibition by exposure to sera and lymphocytes from chimeras, donors and normal dogs. Lymphocytes from sensitized marrow donors inhibited fibroblasts from their chimeras (eight of nine dogs); cell inhibition was abrogated by chimeric serum in only three of eight cases. Only one chimera showed consistent cell inhibition of its "own" fibroblasts, and the serum did not block cell inhibition. The remaining eight chimeras did not show consistent cell inhibition. In conclusion, the cell-inhibition assay is able to detect immunity across "minor" histocompatibility barriers in dogs. Results in chimeras suggest that serum blocking factors are not a prerequisite for maintenance of stable graft-host "tolerance" in DL-A-matched recipients. Similar findings were made in a subsequent study, in which 16 canine marrow-graft recipients were studied sequentially between 50 and 450 days after grafting.

Studies are now in progress involving the use of additional test systems and involving sequential studies of individual patients starting very early after the graft in an attempt to elucidate the complex problem of the "tolerant" state in the human marrow-graft recipient.
Marrow Transplantation from Unrelated Donors

It is obvious from the clinical experiences cited above that a multitude of problems remain to be solved when marrow donor and recipient are siblings matched at the major histocompatibility complex. At the same time, since less than half the patients will have matched siblings, efforts must be directed toward identifying unrelated persons who are perfect matches at the major histocompatibility complex for the patient. With computer technology, and with a large panel of donors, matching for the serologically detected loci can now be accomplished. Rapid advances in the ability to detect and evaluate lymphocyte-detected loci are occurring at present. Thus, we can expect in the near future to be able to identify unrelated donors matched at the major histocompatibility complex, either living donors or cadaver donors whose marrow has been stored. When this goal is achieved, it is important to realize that the unrelated donor matched at the major histocompatibility complex will be no better than the sibling similarly matched. The patient will still encounter the same spectrum of problems described above for the patient with a matched sibling. Hence, it is important to continue to try to solve these clinical and immunologic problems, especially since the vast majority of patients with matched siblings are not now being considered for marrow transplantation.

Discussion

It seems likely that in the near future marrow transplantation will be attempted as therapy for any variety of malignant neoplasia that is sensitive to irradiation or chemotherapy and for non-malignant disorders of the marrow such as thalassemia and sickle-cell disease. At present each new patient with acute leukemia or aplastic anemia should undergo histocompatibility testing along with the members of the family. If the patient has a sibling matched at the major histocompatibility complex, transfusion of blood products should be minimized, and transfusion from family members should be avoided. Marrow grafting can be planned at an appropriate time, perhaps immediately for patients with severe aplastic anemia or acute myelogenous leukemia but only after failure of conventional therapy for patients with mild aplastic anemia and acute lymphoblastic leukemia. Proper selection of the type of patient and the time of grafting raises too many questions to be enumerated here. For example, are the extremely refractory leukemias, described above, the result of prior intensive chemotherapy, and could this problem be avoided by earlier marrow grafting?

Allogeneic marrow grafting in man is still an exceedingly complex and difficult endeavor. Therefore, certain supportive facilities should be considered as mandatory for any center undertaking a marrow-transplantation program, including means of providing the large numbers of platelets and granulocytes that may be required, facilities for the proper administration of total-body irradiation, protection against infection, and bacteriologic monitoring. Laboratory capability should include histocompatibility typing, mixed leukocyte culture and other immunologic studies, production of antithymocyte globulin, cyto- genetics and blood genetic markers. The marrow-graft team, including physicians, nurses and technicians, should have experience in transplantation biology and medicine with a successful program of animal transplantation using the techniques to be applied to man. In this setting the patient should have the maximum opportunity to benefit.

Noteworthy advances in the knowledge of the cause and pathogenesis of human diseases may be expected as a corollary of marrow-transplantation studies. Examples already at hand are the demonstration, by the fact of successful engraftment, that the marrow microenvironment is normal in most patients with aplastic anemia and a demonstration that in vivo malignant transformation of normal human cells can occur in acute lymphoblastic leukemia. Marrow transplantation will also provide insight into many aspects of transplantation biology and tumor immunology. Despite the many discouraging complexities associated with marrow transplantation, some degree of satisfaction may be derived from the fact that knowledge of normal and abnormal immunology and physiology is being increased during endeavors to provide new therapeutic approaches for the patient with otherwise fatal disease.

We are indebted to Dr. Eloise Giblett, who carried out the blood genetic marker studies, and to the entire nursing staff of the Division of Oncology for their management of these patients.

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ACUTE AND CHRONIC
GRaFT-VERSUS-HOST DISEASE

Acute Graft-Versus-Host Disease Following Bone Marrow Transplantation in Humans: Prognostic Factors

M.M. Borit for the Advisory Committee of the International Bone Marrow Transplant Registry

ACUTE GRAFT-V-HOST DISEASE (GVHD) is a major cause of treatment failure following allogeneic bone marrow transplantation in humans. Acute GVHD is a formidable problem even when donors and recipients are perfectly matched as determined by HLA serotyping and by mixed leukocyte culture tests. In this setting moderate to severe acute GVHD occurs in 30% to 70% of patients and is the primary or contributing cause of death in 15% to 40% in most series. In vitro depletion of donor marrow of T cells pretransplant appears to reduce the risk of GVHD. In general, this strategy has not resulted in improved survival experience, however, due to a reduced probability of stable engraftment and, in leukemia patients, an increased probability of recurrent leukemia.

This study was undertaken by the International Bone Marrow Transplant Registry in an effort to identify variables associated with the risk of acute GVHD. In particular, we wished to identify variables that could be manipulated by the referring physician or

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transplant team to reduce the likelihood of this serious complication.

PATIENTS AND METHODS

Data were analyzed from 2,036 consecutive recipients of HLA-identical sibling transplants for leukemia or aplastic anemia who survived at least 31 days with evidence of engraftment and were thus at risk of developing GVHD. Recipients of T cell-depleted bone marrow were not included with the 2,036 patients because there are no generally accepted standards to evaluate the efficacy of T cell removal. The data were provided by 106 bone marrow transplant teams worldwide. GVHD scores were dichotomized into none or mild (grades I to II) v moderate to severe (grades III to IV).

Initially, 33 variables were tested in univariate analyses for associations with the incidence of acute GVHD. Subsequently, variables having an association with a P value ≤ 0.10 in the univariate tests were entered into a multiple logistic equation. In multivariate analyses no significant difference was found in the incidence of GVHD among 661 patients with acute myelogenous leukemia, 509 with acute lymphoblastic leukemia, 369 with chronic myelogenous leukemia, and 390 with severe aplastic anemia. The status of leukemia did not influence the risk of GVHD. Therefore, the four disease groups were combined for the study. Further justification for pooling the data was that no unaccounted for center effects were detected. All P values are based on the results of multivariate analyses.

RESULTS

The incidence of GVHD was 45% (918/2,036), the case fatality rate was 48% (442/918), and GVHD was a primary or contributory cause of death in 22% (442/2,036) of the patients.

The use of alloimmune (via prior pregnancies or transfusions) female donors for male recipients had a significantly higher (relative risk, 2.9; P < .0001) incidence of GVHD than other sex match combinations. Similarly, using nonalloimmune female donors for male hosts was associated with a higher (relative risk, 2.0; P < .001) risk of GVHD than other sex match combinations. These findings suggest that the H-Y male antigen is a target for graft-vs-host reactions and that prior sensitization of female donors to H-Y amplifies the effect.

Transplantation of older patients and/or donors was associated with an increased (relative risk, 1.6; P < .001) incidence of GVHD. The age gradient was modest, however, ranging from 39% in the youngest quartile of patients to 52% in the oldest quartile. Further, the age effect was markedly influenced by the inclusion of alloimmune female-to-male transplants, most of which involved older donors and hosts. Age was no longer a significant risk factor (P > .30) when these 119 transplants were excluded from the analysis.

The incidence of acute GVHD was 65% among the small group of 46 patients who received no prophylaxis against GVHD. This was significantly higher (relative risk, 2.2; P < .01) than the incidence of 44% (542/1,235) in patients receiving methotrexate as prophylaxis against GVHD and the 46% (324/710) incidence in patients receiving prophylactic cyclosporine.

Patients not given trimethoprim sulfamethoxazole in the peritransplant period had a higher incidence of GVHD (50%) than the 44% incidence for those given the drug (relative risk, 1.3; P < .04). Patients whose pretransplant performance rating was less than 90% had a higher incidence of GVHD (49%) than the rate of 44% in those whose performance rating was 90% to 100% (relative risk, 1.2; P < .04).

The occurrence of moderate-to-severe acute GVHD was significantly higher (relative risk, 2.1; P < .0001) among patients given more rather than fewer transfusions posttransplant. It is uncertain, however, whether using large numbers of posttransplant transfusions was the cause of GVHD or a consequence of it.

The estimated probability of GVHD was 16% when none of the adverse factors identified in these studies was present. The probability was 87% to 95% when all of the adverse risk factors were present, with a cumulative relative risk of 5.4 to 5.9.

These studies identified seven factors that had significant associations with the risk of acute GVHD; several of these can be manipu-
lated to reduce the risk. The results should prove helpful in estimating the risk of GVHD for an individual patient. The results also should be of help in designing randomized clinical trials testing different methods to prevent GVHD. For example, if not stratified for the risk factors identified in this study, the rates of GVHD in each arm could vary from 16% to >85% by chance alone.

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Risk factors for acute graft-versus-host disease

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Summary. Acute graft-versus-host disease (GvHD) is an important complication of bone marrow transplantation in humans. Risk factors are imprecisely defined and controversial. We analysed data from 2036 recipients of HLA-identical sibling transplants for leukaemia or aplastic anaemia to identify risk factors for GvHD. Analyses indicate that grading of GvHD can be reproducibly divided into absent or mild versus moderate to severe: 2-year actuarial probability was 54% (95% confidence interval 32–56%) for absent or mild and 46% (44–48%) for moderate to severe. Factors predictive of development of moderate to severe GvHD include donor/recipient sex-match (female—male greater than others, relative risk 2–0. P<0.001). This risk was markedly increased if female donors for male recipients were previously pregnant or transfused (relative risk 2·1, P<0.0001). Older patients were at increased risk of GvHD (relative risk 1·6, P<0·001), but the age gradient was modest, even the youngest patients had a substantial risk of GvHD. If parous or transfused female—male transplants were excluded, age was not a significant risk factor. Cyclosporine or methotrexate were equally effective at preventing GvHD and were superior to no prophylaxis (relative risk 2·3, P<0·01). These data should be useful in estimating the risk of acute GvHD in an individual patient and in designing clinical trials to investigate methods to modify or prevent GvHD.

Graft-versus-host disease (GvHD) is an important complication of bone marrow transplantation in animals and humans (van Bekkum, 1985; Elkins, 1971; Möller, 1985; Sullivan, 1983). GvHD is caused by donor immune cells which probably react against disparate histocompatibility antigens in the recipient. Two forms of GvHD have been described: acute and chronic; these disorders are distinct in timing and clinical features.

Acute GvHD of moderate or greater intensity occurs in 20–80% of recipients of HLA-identical sibling bone marrow transplants. For example, at one institution using similar regimens over a period of years the reported incidence of acute GvHD in various randomized and nonrandomized trials has ranged from 21% to 23% in some studies (Doney et al. 1981a, b; Sanders et al. 1981; Springmeyer et al. 1984; Sullivan et al. 1986) to 70–79% in others (Deeg et al. 1983; Kingemann et al. 1986; Storb, 1983). At another institution the incidence in different sero-ranges from 33% to 34% (Bacchetti et al. 1978; Bräune et al. 1982) to 67–74% (Santos et al. 1983; Tutschka et al. 1980). The reason for this variability in incidence within and between institutions is uncertain and probably reflects several factors including patient selection, differences in diagnosis or grading of GvHD, differences in prophylactic and therapeutic measures, and variability in reporting the cause of treatment failure. One
additional important aspect relates to the inherent statistical variability associated with analyses of relatively small patient series.

Acute GvHD is a major cause of treatment failure following bone marrow transplantation. It is therefore of considerable import to accurately determine risk factors for developing acute GvHD in order to: (1) identify patients at high risk so that special precautions may be taken; (2) identify patients at low risk so that less intense immunosuppressive prophylaxis post-transplant may be given; and (3) to aid in donor selection if more than one is available. To evaluate risk factors for GvHD, we used multivariate statistical techniques to analyse data from 2036 patients reported to the International Bone Marrow Transplant Registry (IBMTR).

METHODS

Included in this analysis were 661 patients with acute myelogenous leukaemia (AML), 589 with acute lymphoblastic leukaemia (ALL), 396 with chronic myelogenous leukaemia (CML) and 390 with severe aplastic anaemia who received bone marrow transplants between 1 January 1978 and 30 June 1985. All patients received transplants from allogeneic HLA-identical sibling donors and survived ≥ 21 d with evidence of engraftment. 106 transplant teams worldwide reported their consecutive transplant experience. Data were analysed as of 31 January 1986: minimum follow-up was 7 months, maximum follow-up 7 years. During this interval, 909 additional leukaemia and severe aplastic anaemia patients were reported to the IBMTR, but were excluded from this study: 12 receiving fetal cells or tissues; 79 receiving bone marrow from genetically identical twins; 442 receiving bone marrow from donors other than HLA identical siblings; 98 dying < 21 d post-transplant who could not be evaluated for acute GvHD; 64 surviving ≥ 21 d without evidence of engraftment; and 214 recipients of bone marrow that was treated in vitro to deplete T cells. The latter were excluded at this time for several reasons. This approach is new and more than 15 different techniques are in use with resultant small group sizes. Further there are no widely accepted criteria to measure the efficiency of T cell removal and evaluation of its effect on the incidence and severity of acute GvHD depends on whether the technique was successful.

For purposes of this investigation, potential risk factors were grouped into three categories: recipient variables, transplant variables and post-transplant variables. A partial list is presented in Table I.

GvHD was evaluated by previously reported, prospectively defined criteria recommended by the IBMTR (Advisory Committee, 1975). Briefly, involvement of skin, liver and gastrointestinal tract were scored absent, mild, moderate, moderately severe or severe and an overall score was generated.

Thirty-three potential prognostic variables were studied. The association between each factor and acute GvHD was tested using χ², Wilcoxon rank sum or t tests. Factors found to be associated with acute GvHD with a P value ≤ 0.10 in these univariate tests were examined for the presence of interactions with a two-way analysis of variance test. The resulting factors and the significant interactions (P < 0.05) were included in a stepwise multivariate logistic model. From the logistic analysis, we derived the probability of acute GvHD for a patient with a single adverse risk factor or a specific profile of adverse risk factors. The relative risk associated with a single risk factor or profile was computed as the probability of GvHD for patients with that risk factor or profile divided by the probability of GvHD for patients with none of the adverse risk factors. The rates of GvHD among diseases (42% in ALL, 43% in severe aplastic anaemia, 45% in AML and 53% in CML) were not significantly different then all significant risk factors were entered in a forward stepwise multiple logistic regression analysis. To evaluate whether any single disease affected the conclusion, a jackknife approach (Mosteller & Tukey, 1977) was used to determine if the inferences about the risk factors would be affected by exclusion of any of the four disease categories. That is, all the same significant risk factors were tested when the analysis was repeated on each of the subsamples that had one of the disease categories excluded. Since the results passed this test for internal consistency over disease category, the results with the most statistical power—combining all four diseases—are pre-

| Table I. Key factors that were tested for associations with the risk of moderate to severe acute graft-versus-host disease |
|--------------------------------------------------|---------------------|------------------------------|
| Recipient variables | Transplant variables | Post-transplant variables |
| Diagnosis | Year of transplant | Antibiotic deconjugation |
| Status of disease | Conditioning regimen | Trimethoprim–sulfamethoxazole |
| Age | Age of donor | Type of isolation |
| Sex | Sex of donor | Prophylaxis of GvHD |
| Coexisting disease | Allimmune donor* | Number of transfusions |
| Performance rating pretransplant | ABO match | |
| Interval diagnosis to transplant | Sex match | |
| Number of pretransplant transfusions | Dose of bone marrow cells | |

* Previously pregnant or transfused.
RESULTS

Analysis of GvHD grading
Before determining factors predictive of acute GvHD, it was necessary to validate reporting criteria from participating centres. This was performed as follows. A hypothetical index profile was selected consisting of patients with the following features: acute leukaemia. HLA-identical sibling donor, pretransplant conditioning with cyclophosphamide (< 173 mg/kg body weight), total body radiation (8-12 Gy) and posttransplant immune suppression with methotrexate or cyclosporine to prevent GvHD. GvHD data for 684 patients who fulfilled these criteria from 69 of the 106 centres of varying size were compared. The incidence of no or mild GvHD was relatively consistent, 57% with a 95% confidence interval of 52-62% within this group. The distribution of patients with no versus mild GvHD varied somewhat between 22 centres that reported 10 or more patients. When we compared GvHD scores among 47 teams that reported fewer than 10 with the 22 teams that reported 10 or more patients, we found that absent or mild scores were assigned in 57% by both the smaller and larger centres. Since no significant distinction was detected between no or mild GvHD, these two groups were combined in the analyses.

Likewise, the incidence of moderate to severe acute GvHD was relatively consistent in the 69 centres: 43% with a 95% confidence interval of 38-48%. Within this group, the distribution of cases with moderate versus moderately severe versus severe acute GvHD varied but there was no consistent difference based on geographic location or centre size. Because of the relative consistency of GvHD reporting, we divided the analysis of GvHD into none or mild versus moderate to severe GvHD.

Risk of GvHD. The incidence of moderate to severe acute GvHD was 45 ± 2% (95% confidence interval) (918/2036) and the actuarial probability at 6 months was 46 ± 2%. 48% (442/918) of patients who developed moderate to severe acute GvHD died with this complication. Overall, GvHD was a primary or contributory cause of death in 22% (442/2036) of the patients in this study. Variables significantly associated with the risk of GvHD are shown in Table II.

Sex and sex match. Neither recipient nor donor sex was significantly associated with GvHD. The incidence of GvHD in male recipients was 48 ± 3% versus 41 ± 3% in female recipients. Similarly, GvHD in recipients of cells from male donors was 47 ± 3% versus 44 ± 3% in recipients of cells from female donors.

Female—male grafts had a higher incidence of GvHD (53 ± 4%) than the three other types of matches (42 ± 2%); none of the latter differed significantly from one another. Because prior pregnancies or transfusions might affect the likelihood of cells from female donors to cause GvHD, we investigated the incidence of GvHD in recipients of transplants from females with or without prior pregnancies or transfusions. This variable had no statistically significant effect on the risk of GvHD when the recipient was female: 39 ± 9% versus 34 ± 6%, respectively. When the female donor, was not previously pregnant or transfused, female-
presented. Differences in the risk of GvHD between treatment centres that could not be accounted for by recorded information were tested in two ways in logistic analysis after adjusting for the known risk factors. First, we compared the group of patients transplanted in the seven largest centres with the group from the smaller centres. Second, we compared rates of acute GvHD in patients from each of the seven largest centres with the smaller centres. The actuarial incidence of acute GvHD was computed using standard life table methods.

**STUDY GROUP**

Recipient variables. Data from 2036 recipients of HLA-identical bone marrow transplants were analysed. There were 1192 males and 844 females; median age was 21 years (range <1–59). 777 (47%) of 1646 patients with leukaemia were transplanted in first complete remission or chronic phase. 20% in second complete remission and 33% with more advanced disease. In patients with acute leukaemia, CNS leukaemia was present pretransplant in 7% and 9% had one or more chromosome abnormalities at the time of diagnosis. Coexisting diseases were present in 11% of patients. Median performance score (Karolinsky et al. 1948) pretransplant was 100% (range 10–100). Median interval from diagnosis to transplant was 9 months (range <1–195). 58% of transplants were performed within 1 year and 76% within 2 years of diagnosis. Median number of pretransplant transfusions was 14 (range 0–99); patients with CML received the fewest transfusions (median <1), those with ALL, an intermediate number (median 10), and those with aplastic anaemia (median 20) and AML (median 25) the most (P < 0.0001). 32% of patients with CML had a splenectomy prior to transplantation.

Transplant variables. Donors were male in 1111 cases and female in 925. Median donor age was 22 years (range <1–62). 1065 transplants were sex matched (male = male, 33%; female = female, 20%) and 971 sex mismatched (male = female, 22%; female = male, 26%). Among female donors, 231 had been pregnant or had received transfusions; 517 had not; and information regarding parity or transfusions was either not available or not reported for 177. 68% of transplants were ABO identical and 32% were incompatible; of these 325 were mismatched with regard to graft rejection (e.g. A = O) and 285 with regard to GvHD (e.g. O = A). Most (1816) patients were prepared for transplantation with chemotherapy (cyclophosphamide, cytarabine, daunorubicin, vincristine and others) and radiation; 213 received chemotherapy alone and seven radiation alone. Most patients who received chemotherapy alone had severe aplastic anaemia. Doses of radiation ranged from 3 to 16 Gy given in 1-20 fractions at dose rates of 1-3 to 99 cGy/min using either 1r or X-irradiation. Radiation fields included total body with or without lung shielding for 1695 patients and total lymphoid, total nodal, or thoraco-abdominal radiation for 128 patients. Median dose of unmanipulated bone marrow cells transplanted was 3.1 × 10⁹/kg (range 0.2–18.8). Oral antimicrobials were given pretransplant for decontamination of the gastrointestinal tract in 76% of the patients.

Post-transplant variables. Post-transplant prophylaxis of GvHD consisted of methotrexate in 1235 patients and cyclosporine in 710. Both methotrexate and cyclosporine were given to 29 patients. 16 received antithymocyte globulin, corticosteroids, cyclophosphamide or other agents and 46 received no prophylactic treatment for GvHD. Median number of post-transplant transfusions was 18 (range O–99); only 15 (0.7%) patients received unirradiated transfusions post-transplant and these patients were analysed separately. Most patients (60%) were managed in conventional isolation, or in laminar airflow environments (38%); 2% were not isolated. Trimethoprim–sulfamethoxazole for prophylaxis of Pneumocystis carinii and/or bacterial infections was used in 72% of patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lower risk</th>
<th>Higher risk</th>
<th>Relative risk</th>
<th>P</th>
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<td>Donor-recipient sex-match</td>
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<td>Allo 9-9</td>
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<td>&lt;0.01</td>
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<td>Donor-recipient sex-match</td>
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<td>Not Allo 9-9</td>
<td>2-0</td>
<td>&lt;0.001</td>
</tr>
<tr>
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<td>Younger</td>
<td>1-6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Trimethoprim–sulfamethoxazole</td>
<td>Given</td>
<td>Not given</td>
<td>1-3</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Pretransplant performance rating</td>
<td>Higher</td>
<td>Lower</td>
<td>1-2</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>Post-transplant transfusions†</td>
<td>Less</td>
<td>More</td>
<td>2-1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* Allo refers to female donors with previous pregnancies or transfusions; Not Allo refers to female donors who had not been pregnant or transfused.
† It is not clear whether the association between large numbers of post-transplant transfusions and an increased incidence of acute graft-versus-host disease represents a causal relationship or is the effect of existing graft-versus-host disease.
Risk Factors for Acute GvHD

Figure 2. Multivariate cumulative relative risk (numbers in parentheses) and cumulative percent probability of moderate to severe acute graft-versus-host disease. When one of the significant adverse risk factors identified in this study was present, a risk of 1.0 was used. First adverse risk factor = alloimmune female—male transplant; second = no prophylaxis against graft-versus-host disease; third = older patients; fourth = trimethoprim—sulfamethoxazole not given; fifth = lower pretransplant performance ratings; sixth = larger number of post-transplant transfusions.

patients with severe aplastic anaemia, prior splenectomy, presence of infection in the week prior to transplantation, bone marrow cell dose, use of unirradiated transfusions post-transplant (note: only 15 patients were available for study), use of pretransplant decontamination of the gastrointestinal tract and conventional versus laminar airflow isolation. No 'centre effect' was detected.

DISCUSSION

Moderate to severe acute GvHD occurred in 45 ± 2% of the 2036 patients receiving bone marrow transplants from HLA identical siblings. The strongest predictive factor having an adverse effect on the risk of GvHD was the use of previously pregnant or transfused female donors for male recipients. The risk of GvHD also was significantly increased when female donors who had not been pregnant or transfused were used for male recipients in comparison with other sex-match combinations. The IBMTR previously reported that female—male transplants were associated with an increased risk of GvHD in 48 patients with severe combined immunodeficiency disease (Bor tin & Rimm, 1977). Female—male transplants also had an increased incidence of GvHD in 264 patients with leukaemia reported by Zwaan & Hermans (1982). Some but not all experimental data in mouse (Lengerova & Chutna, 1959; Uphoff, 1975) using nulliparous or multiparous donors support the finding that the risk of GvHD is increased in female—male transplants.
The relationship between donor/recipient sex and sex-matching with GVHD is controversial. For example, in several reports the incidence of GVHD was elevated when female donors were used irrespective of the sex of the recipient (Atkinson et al. 1986; Borton et al. 1981; Ellenbein et al. 1983; Gluckman et al. 1981). Neither sex of the donor nor recipient was associated with the risk of GVHD in this study. In other studies, both female—male and male—female sex-mismatched transplants had an increased incidence of GVHD (Bross et al. 1984; Storb et al. 1977, 1984). In this study the risk was highest in female—male transplants (53 ± 4%) and significantly lower in the other sex-match combinations: female—female (39 ± 5%) male—male (44 ± 4%) and male—female (42 ± 5%). Because of the finding that use of female donors who were parous or transfused was associated with a high incidence of GVHD in male (but not female) recipients, we wished to examine whether the sex of the progeny of parous females or sex of their blood donor(s) affected the likelihood of GVHD, but these data were not available.

The relationship between age and acute GVHD is complex and controversial. We and others reported that recipient and/or donor age correlates directly with the risk of GVHD (Bordin et al. 1981; Gluckman et al. 1981; Ringden & Nilsson, 1985; Storb et al. 1977, 1983; Weiden, 1980; Zwaan & Hermans, 1982). In the present study we again found a direct correlation between age and the risk of GVHD (P < 0.001). This relationship was biased, however, by inclusion of the 119 males receiving transplants from previously pregnant or transfused female donors, almost all of whom were in the older age group. Patient age was not significant when this group was excluded from analysis.

It has been suggested that GVHD is infrequent in very young patients (Ramsay et al. 1982). This notion is not supported by our data. For example, the incidence of moderate to severe acute GVHD was 33% in patients 0–6–6–6 years of age. Age did, however, have a substantial impact on mortality from GVHD: GVHD was a primary or contributory cause of death in 26% of individuals in the youngest quartile who developed GVHD versus 44% of individuals in the oldest quartile with GVHD (P < 0.0001).

The relative efficacy of cyclosporine and methotrexate in preventing GVHD is controversial. In non-randomized and randomized studies the risk of GVHD was significantly increased with cyclosporine in some (Barrett et al. 1982; Deeg et al. 1983; Speck et al. 1983) but not all reports. No significant difference between the efficacy of cyclosporine and methotrexate on the risk of GVHD was detected in this study. A detailed analysis of individuals receiving T-cell depleted transplants, a combination of methotrexate and cyclosporine, or methotrexate or cyclosporine combined with corticosteroids will be published elsewhere.

There is controversy as to whether methotrexate is superior to no prophylaxis (Lazarus et al. 1984, Sullivan et al. 1986). In this study the incidence of GVHD was significantly increased in patients given no prophylaxis versus those given methotrexate or cyclosporine.

Three variables were noted to have a marginal association with the risk of GVHD: use of prophylactic trimethoprim–sulfamethoxazole, pretransplant performance status and number of post-transplant transfusions. In animals, intestinal decontamination or modification of the intestinal flora is associated with a decreased risk of GVHD (van Bekkum et al. 1974). We found that the risk of GVHD was somewhat reduced in patients given trimethoprim–sulfamethoxazole pretransplant (44 ± 3% versus 50 ± 4%, P < 0.04). The explanation for this difference is unknown. Interestingly, patients who received other antibiotics for the purpose of intestinal decontamination had the same risk of GVHD as patients not receiving decontamination. Thus it is unlikely that differences in the intestinal microbial flora are responsible for the decreased risk of GVHD observed in patients receiving trimethoprim–sulfamethoxazole.

Patients who had the highest performance ratings immediately prior to transplantation had a somewhat lower risk of GVHD compared to patients whose performance ratings were less than 50% (44 ± 3% versus 49 ± 5%, P < 0.04).

The association between the number of transfusions administered post-transplant and the risk of GVHD is difficult to interpret. It is unclear whether the association was cause or effect. Although unirradiated transfusions of whole blood or blood components such as platelets or granulocytes are capable of causing lethal GVHD in immunocompromised patients (von Frieder et al. 1982; Nikoskelainen et al. 1983; Weiden, 1984), essentially all patients received only irradiated transfusions post-transplant. Intravenous administration of spleen cells irradiated with 10 Gy is associated with an increased risk of GVHD in immunosuppressed rodents (Bennet & Hand, 1978). Thus, it is possible that the number of irradiated post-transplant transfusions correlated directly with the risk of acute GVHD because radioresistant cells capable of inducing GVHD in immunosuppressed patients are present in irradiated blood products. Another possible explanation relates to the observation that transfusions are correlated with an increased risk of CMV infection (Weiner et al. 1986; Winston et al. 1980) which, in some animal models and man, has been associated with an increased risk of GVHD (Grundy et al. 1985). On the other hand, GVHD has been reported to activate latent CMV infections (Dowling et al. 1977). The incidence of CMV interstitial pneumonitis was 14 ± 2% in patients with moderate to severe acute GVHD versus 6 ± 1% in patients with no or mild acute GVHD (P < 0.0001). It is also possible that the critical clinical status of patients with moderate to severe acute GVHD necessitated large numbers of transfusions and that the correlation was the result of GVHD rather than its cause. Additional studies, including the dose of radiation to the blood and the precise relationship between the onset of GVHD and timing of transfusions, are needed to resolve this question.

Several factors reported to be associated with the risk of GVHD in smaller series of patients were not confirmed in this study. Storb et al. (1983) found a reduced risk of GVHD in severe aplastic anemia patients who were maintained in a protective environment: the same team found no association in other studies (Anasetti et al. 1986; Buckner et al. 1978; Storb et al. 1986). We found no significant difference in the incidence of GVHD among patients maintained in laminar airflow isolation versus conventional isolation.

There is debate whether different diseases are associated
with different risks of developing GvHD. Bross et al (1984) reported that the risk of GvHD was highest in patients with acute lymphoblastic leukaemia in comparison with those with other diagnoses. An editorial (1984) suggested that the incidence of GvHD is highest in severe aplastic anaemia. We found no differences in multivariate analysis.

Most data suggest that ABO incompatibility is not a risk factor for GvHD (Buckner et al. 1978; Gale et al. 1977; Hershko et al. 1980). In contrast, major and minor ABO mismatches were reported to be associated with GvHD by Bacigalupo et al (1985) in 109 transplanted patients. No significant associations were found between major and minor ABO mismatches and GvHD in the present study.

In animal models, the incidence and severity of GvHD is increased when total body radiation rather than chemotherapy is used for pretransplant conditioning (Elkins. 1971). Also, different doses, schedules and fields of radiation were reported to produce different rates of GvHD in animals (Gottlieb et al. 1980; Slavin et al. 1978). We found no significant difference in the incidence of GvHD with different conditioning regimens.

Data from animal models support the concept that the number of cells transplanted correlates directly with the incidence of GvHD (van Bekkum. 1983). In patients with AML transplanted in first remission, the IBMTR (Bortin et al. 1983) found a trend toward an inverse correlation between bone marrow cell dose and the risk of GvHD. Ringden & Nilsson (1985) found a significant (P<0.01) inverse relationship between cell dose and the risk of GvHD. Atkinson et al (1986) found no effect of cell dose. numbers of T cells or T cell subsets transplanted and the risk of GvHD. In this study there was no significant effect of cell dose on the risk of GvHD.

The reason for the discrepancies between the findings of this study and others is unclear. In some instances other reports were based on relatively small sample sizes of less than 300 patients. In other cases, failure to identify significant risk factors. even in randomized clinical trials, may have been due to small sample sizes with substantial type II (β) errors. often in excess of 80%. Certainly, one important aspect of this study is the use of pooled data from more than 2000 consecutive transplants reported by more than 100 teams. It is unlikely that several of the positive and negative associations reported here could have been identified in smaller databases.

The data presented in this study indicate that GvHD remains a major problem following bone marrow transplantation from HLA-identical siblings. Although the risk of GvHD may be reduced by T-cell depletion of donor bone marrow in vitro (for review, see Gale, 1987), this approach is associated with other problems such as an increased risk of graft rejection and leukaemia relapse (Gale & Reirner. 1986). Other approaches such as the combination of methotrexate and cyclosporine or either drug combined with corticosteroids may also be useful.

In this study we identified several prognostic factors, some of which can be manipulated in a favourable direction. These results should be useful in estimating the risk of GvHD in individuals with a specific profile of risk factors. The data also should prove useful in developing future approaches and in designing prospective clinical trials testing alternative methods to prevent GvHD. For example, if the randomization is not stratified for the prognostic variables identified here, it would be possible to observe rates of moderate to severe acute GvHD of 16% to >85% by chance alone in either or both arms.


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Major Complications of Marrow Harvesting for Transplantation

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Complications to bone marrow donors were analyzed for 2027 transplants reported to the International Bone Marrow Transplant Registry and for 1263 transplants performed in Seattle. Thus, a total of 3290 donor risk episodes were available for study. The incidence of major, i.e., life-threatening complications in the combined series was 0.27%. Neither death nor permanent residua occurred in any of the donors.

Marrow transplantation is increasingly performed as a therapeutic modality. Donors usually have been HLA identical siblings but improving results have encouraged extension of the donor pool to include less than perfectly matched family members (1) and fully matched unrelated donors (2). It can be anticipated that there will be a further increase in marrow transplantation in the next several years and this will result in a further intensification of the search for acceptable donors.

The decision to perform a marrow transplant involves weighing the risks and benefits to the patient, as well as the risks to the donor. There have been numerous reports presenting risk-benefit ratios as they affect the marrow recipient, but there is essentially no literature describing the risks to the donor.

The purpose of this paper is to present the major, i.e., life-threatening complications that have occurred to marrow transplant donors as reported to the International Bone Marrow Transplant Registry (IBMTR) together with those
that have occurred in donors aspirated by the Seattle Marrow Transplant Team (SMTT). More detailed reports describing less serious complications and statistics relating to the procedure will be forthcoming from the IBMTR and the SMTT.

MATERIALS AND METHODS

From its inception in 1970 through June 30, 1983, reports of 2248 allogeneic or syngeneic transplants have been submitted to the IBMTR by 99 bone marrow transplant centers, worldwide. In this report we have excluded from the IBMTR analysis reports of 176 fetal tissue or thymic epithelial transplants, 10 bone marrow transplants in which the response to the question regarding donor complications was not completed and, to avoid duplication, 35 bone marrow transplants reported to the IBMTR by the SMTT. Thus, there were 2027 instances in the IBMTR series in which bone marrow donors were at risk of complications attendant to the procedure.

The hospital records of 1160 allogeneic or syngeneic donors of marrow in Seattle were reviewed and the complications of 1263 separate donations analyzed. This report includes the majority, but not all, of the donors aspirated by the SMTT. Only records of donors aspirated at the transplant center were analyzed, including all marrow aspirations at the USPHS Hospital (1969–73), Providence Medical Center (1973–75) and the Swedish Hospital Medical Center (1975–Dec 31, 1982). The only exclusions from Seattle were procedures performed at other hospitals and patients aspirated for autologous transplantation.

When a donor gave marrow on two or more occasions, each instance was considered separately for risk of complications. Thus, from both series there was a total of 3290 bone marrow donations, each representing an individual risk episode. Marrow aspirations almost always were performed from the posterior iliac crests (3). In addition, the anterior iliac crests often were aspirated and occasionally the sternum. Almost all patients had general or spinal anesthesia; caudal blocks were used infrequently.

RESULTS

The IBMTR series includes three episodes of life-threatening marrow donor complications. A brief description of each such complication is presented below:

IBMTR ID 184, a 10-yr-old boy had marrow aspirated from the posterior iliac crests under general anesthesia. He developed fever and blood cultures revealed Enterobacter species. He was treated with gentamycin. Recovery was prompt and complete.

IBMTR ID 1358, a 60-yr-old female underwent marrow aspiration from the posterior iliac crests under general anesthesia. She developed a femoral vein thrombosis at the site of an indwelling femoral vein catheter. Treatment with streptokinase was complicated by a small retroperitoneal hemorrhage that was identified on CAT scan. Transfusions were not required and recovery was complete without sequelae.

IBMTR ID 1765, a 53-yr-old female developed a shortness of breath shortly after marrow aspiration. A roentgenogram of the chest was compatible with a small pulmonary embolus with atelectasis. She was treated with heparin and oral anticoagulants. The response was prompt and recovery was complete within 2 wk.

The following life-threatening events occurred in the SMTT experience:

SMTT UPN 50, a 19-yr-old male underwent marrow aspirations under general anesthesia from the anterior and posterior iliac crests. On extubation, he was noted to have bronchospasm and a chest X-ray was compatible with aspiration pneumonitis. He was treated with prednisone and antibiotics and discharged after 5 days with a normal chest X-ray.

SMTT UPN 561, a 26-yr-old female received preoperative medication consisting of morphine 10 mg and scopolamine
0.4 mg IM. One h later, she was given Valium 5 mg IV followed in 5 min by instillation of Pontocaine 12 mg intrathecally followed by Valium 5 mg IV. Thirty min later, shortly after commencement of the marrow aspiration procedure, the patient suffered a cardiopulmonary arrest which responded to external cardiac massage and the administration of epinephrine and Decadron. There were no sequelae. Marrow aspiration was carried out 24 h later under general anesthesia without complication.

**SMTT UPN 1085**, a 4-yr-old male underwent marrow aspirations from the posterior iliac crests under general anesthesia with Halothane. Ten min into the procedure, he developed sinus tachycardia, then developed ventricular tachycardia. The procedure was halted, the patient was turned and a blow was administered to the precordium. He reverted to normal sinus rhythm with intermittent ventricular irregularities which responded to Lidocaine. Subsequent electro- and echocardiograms were normal and there were no sequelae.

**SMTT UPN 1493**, a 43-yr-old man was evaluated on August 31, 1981 as a potential marrow donor. He was found to have hypertension with a blood pressure of 160/110 mm Hg and placed on Lopressor and diuretics. On September 17, 1981 he was admitted for marrow aspiration and his blood pressure was 150/100 mm Hg. The following day, he underwent multiple marrow aspirations from the anterior and posterior iliac crests and the sternum under general anesthesia without complications. On February 1, 1982 he was readmitted for a second marrow donation and underwent marrow aspirations from the anterior and posterior iliac crests without problems. His blood pressure prior to and during the procedure was in the normal range. On the evening of discharge, he developed paresis of the left side of his body. A CAT scan was consistent with a cerebral infarction with a right middle cerebral distribution. Electroencephalogram showed temporal slowing and an echogram showed stenosis of the carotid bifurcation on the right. In March of 1982, he underwent a carotid endarterectomy and at the time of this writing, July 1983, he has made an almost complete recovery.

**SMTT UPN 1641**, a 44-yr-old man developed tenderness over the posterior iliac crests 24 h after the procedure. He was treated empirically with Dicloxicillin and, when cultures of the aspiration sites revealed *Enterobacter cloacae*, with Trimethoprim-Sulfamethoxazole. He was discharged from the hospital but because of persistent infection he was subsequently readmitted for 10 days to receive intravenous antibiotics. In addition, he required incision and drainage of the infected area and had an uneventful outcome.

**SMTT UPN 1900**, a 60-yr-old man underwent marrow aspirations from the anterior and posterior iliac crests under spinal anesthesia. He was noted to be febrile on return to the ward and one of four blood cultures was positive for *Klebsiella pneumonia*. He was treated with intravenous antibiotics for 2 wk and discharged without sequelae. Intensive studies failed to reveal a source for the bacteremia.

**DISCUSSION**

Life-threatening complications occurred in 3 of 2027 IBMTR donations and 6 of
1263 donations in the SMTT experience for an overall incidence of 0.27%. There was no threat to life in 99.73% of the marrow donations. Although essentially all donors had pain and soreness at the aspiration sites for several hours to several days following the procedure, pain that persisted for a week or more occurred in less than 0.5% of the donations. Other consequences of marrow donation included anemia of sufficient magnitude to require transfusions in a few instances, one case of a broken aspiration needle that required surgical removal, and a few transient episodes of hypotension, atrial arrhythmia or laryngospasm.

Five of the life-threatening complications reported here (non-fatal cardiac arrest, pulmonary embolus, aspiration pneumonitis, ventricular tachycardia and cerebral infarction) probably could be attributed to use of a general anesthetic. The occurrence of these life-threatening complications associated with general anesthesia, while rare, was anticipated from experience accumulated in healthy individuals undergoing surgical procedures (4). Although no deaths occurred in either the IBMTR or SMTT series, the authors are aware of a fatality in an older donor at a transplant center that has not reported any of their cases to the IBMTR. While the probability of an anesthetic death is exceedingly small in healthy individuals of any age, the risk is highest in the elderly (4). It should be noted that less than 1% of all donors in the IBMTR and SMTT series were over 60 yr of age.

The potential for fatal complications resulting from marrow aspiration per se is remote and probably limited to the consequences of sepsisemia or infection at the aspiration sites as occurred in three cases reported here.

These data demonstrate that marrow donation for transplantation is an exceedingly safe procedure but with finite and definable risks. Information on donor complications should continue to be accumulated carefully and disseminated widely.

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Bone marrow transplant centers that contributed donor data for this report

Institute of Medicine, Adelaide, Australia
Royal Children’s Hospital, Victoria, Australia
Royal Hobart Hospital, Tasmania, Australia
Royal Perth Hospital, Perth, Australia
St. Vincent’s Hospital, Darlinghurst, Australia
Westmead Centre, Westmead, Australia
Med. Univ.-Klinik, Vienna, Austria
Academisch Ziekenhuis, Leuven, Belgium
Cliniques U. St-Luc, Bruxelles, Belgium
U. Catholique de Louvain, Bruxelles, Belgium
McMaster University, Hamilton, Canada
Montreal Children’s Hospital, Montreal, Canada

1 The donor, a male in his mid-sixties, suffered a cardiac arrest during induction of general anesthesia; all efforts to restore cardiac function were unsuccessful (Prof. U. W. Schaefer, personal communication).
Princess Margaret Hospital, Toronto, Canada
Royal Victoria Hospital, Montreal, Canada
Rigshospitalet, Copenhagen, Denmark
Royal Free Hospital, London, England
Royal Marsden Hospital, London, England
Rigshospitalet, Oslo, Norway
Victoria Infirmary, Glasgow, Scotland
Ciudad Sanitaria, Valencia, Spain
University of Barcelona, Barcelona, Spain
Research Centre, Cape, South Africa
Huddinge University, Huddinge, Sweden
Clin. Infantlle University, Lausanne, Switzerland
Kantonsspital, Basel, Switzerland
Kinderspital, Zurich, Switzerland
University Hospital, Zurich, Switzerland
Albany Medical College, Albany, USA
Arkansas Children’s Hosp., Little Rock, USA
Baylor College of Medicine, Houston, USA
Bishop Clarkson Memorial Hospital, Omaha, USA
Cancer Research Inst., San Francisco, USA
Case-Western Reserve University, Cleveland, USA
Cedars-Sinai Med. Ctr., Los Angeles, USA
Children’s Hospital, Philadelphia, USA
Children’s Hospital, Cincinnati, USA
Cleveland Clinic, Cleveland, USA
Cornell Medical Center, New York City, USA
Duke University Med. Ctr., Durham, USA
Emory University, Atlanta, USA
Hahnemann Hospital, Philadelphia, USA
Johns Hopkins University, Baltimore, USA
Loyola University, Chicago, USA
M.D. Anderson Hospital, Houston, USA
Marshfield Clinic, Marshfield, USA
Massachusetts General Hospital, Boston, USA
Memorial Sloan-Kettering Inst., NYC, USA
Michigan State U., East Lansing, USA
Mount Sinai Medical Center, Milwaukee, USA
Mount Sinai Medical School, NYC, USA
National Cancer Institute, Bethesda, USA
National Jewish Hospital, Denver, USA
N. Carolina Mem. Hosp., Chapel Hill, USA
Northwestern University, Chicago, USA
Oak Ridge Associated U., Oak Ridge, USA
Oklahoma Teaching Hospitals, Oklahoma City, USA
Oregon Health Sciences U., Portland, USA
Pacific Med. Center, San Francisco, USA
Roswell Park Memorial Inst., Buffalo, USA
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ANNOUNCEMENT

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Monograph Number 2

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This proceedings contains reports on the structure and function of the lymphatic system, including: Cellular migration streams, integration of the lymphomyeloid complex; Lymphocyte production and differentiation; Therapeutic approaches to the lymphatic system; Therapy for lymphedema; Imaging of the lymphatic system; Anatomy, physiology and pathology of the lymphatic system; Use of monoclonal antibodies in medicine; and other topics. Monograph No. 2, 1984, $25.00 US advance payment includes book rate mail delivery, for air mail delivery additional $7.00. Make check drawn on U.S. bank payable to: Immunology Research Foundation, Inc.

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ORIGINAL ARTICLE

Cognitive, educational, psychosocial adjustment and quality of life of children who survive hematopoietic SCT and their siblings

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Our objective was to compare cognitive, educational and psychosocial outcomes, and quality of life (QOL) of pediatric hematopoietic SCT (HSCT) survivors with those of their siblings, 2 years post-HSCT. Forty-six HSCT survivors, with age ranging from 3 to 16 years, and 33 siblings, with age ranging from 3 to 20 years, participated. Standardized tests were performed and questionnaires were completed by the participating children and their mothers. Survivors’ full, verbal and performance IQ scores did not differ significantly from those of their siblings. Survivors, however, had significantly higher perceptual organization scores than their siblings. Siblings’ mean scores on spelling were significantly higher than those of survivors, but arithmetic and reading scores were not. Siblings had significantly more internalizing problems than survivors. Siblings’ physical QOL scores were significantly better than those of survivors. Finally, child age, maternal depression scores and age, and family cohesion were related to cognitive and educational differences. A history of cranial radiation and a diagnosis of neuroblastoma or Hodgkin’s lymphoma in survivors were related to the difference in internalizing scores. Except for some deficits in educational outcomes and physical QOL, survivors’ cognitive and psychological outcomes at 2 years post-HSCT were similar to those of their siblings. Family and clinical factors were identified as critical for these outcomes.

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Keywords: cognitive; educational; psychosocial outcomes; children; HSCT

Introduction

Children and adolescents who undergo hematopoietic SCT (HSCT) may be vulnerable to cognitive, educational, perceptual motor skills and psychosocial deficits resulting from the treatment and disease. Although early retrospective studies have reported cognitive and academic deficits in survivors of pediatric BMT, particularly if they received cranial radiation at a younger age, more recent prospective studies have found that, in general, survivors of HSCT perform well, cognitively and educationally, or even improve some of their performance 2 years post-HSCT compared with their pre-HSCT performance. Moreover, some child characteristics (older age at transplant) and familial factors (older maternal age and less maternal depression symptoms) seem to be associated with better outcomes. It is less clear how survivors’ outcomes compare to those of their healthy siblings, who are exposed to the family crisis precipitated by the diagnosis of a life-threatening illness such as cancer, the intense treatment that culminates in a transplant and related complications. This study addresses this gap.

Survivors of childhood cancer have been compared with their close-in-age siblings in cognitive, educational, quality of life (QOL) and psychosocial outcomes with variable results. Indeed, some studies have found that survivors have more cognitive and educational deficits than their siblings. Other studies have found no differences between survivors of childhood cancer and their healthy siblings in behavioral, educational and social problems, as well as feelings of distress, guilt and psychosomatic symptoms. In contrast, in an early study, siblings of childhood cancer survivors showed more distress than the survivors in perceived social isolation; or survivors performed like their siblings in psychological adjustment but did significantly worse than their siblings in educational outcomes. A recent study examining post-traumatic stress found that siblings reported more symptoms of post-traumatic stress than did childhood cancer survivors. In another recent study, siblings and survivors of leukemia did not differ in behavioral problems but siblings displayed more social and academic difficulties. Clearly, the current inconclusive evidence comparing children with cancer with their siblings provides little direction as to whether survivors of HSCT will be worse off than their siblings.

To date, to our knowledge, there are no published studies that compare the comprehensive outcomes of pediatric cancer survivors and their healthy siblings.
HSCT survivors with those of their siblings. The only comparative study of siblings in this population examined the psychosocial outcomes of donor and non-donor siblings of children who undergo HSCT, but did not compare them with the ill child. Donors were reported to have significantly more anxiety than non-donors but non-
donors were reported to have more problems at school. HSCT inflicts added stress on children and families already overburdened with a wide range of medical, psychosocial and financial stresses related to the diagnosis of cancer or other blood disorders and unsuccessful treatment. Considering this and the aggressiveness of the HSCT procedure, one may expect that the outcomes for survivors and siblings may be worse than the outcomes for childhood cancer survivors who do not undergo an HSCT. Augmenting the knowledge of the long-term cognitive, educational, psychosocial and overall QOL outcomes of survivors will help clinicians and families to make decisions regarding comprehensive care and future planning.

Thus, the main objective of this study was to compare cognitive, educational, perceptual motor skills, and psychosocial outcomes and QOL of HSCT survivors with their close-in-age siblings at 2 years post-HSCT. The secondary objective was to examine clinical, familial and child factors that may be associated with the differences, if any, between the groups. To examine related factors, we used a conceptual model to explore the potential factors that could explain any of the differences between the outcomes for survivors and siblings, which was similar to that used in earlier studies of children who were diagnosed with cancer, their parents and the longitudinal sample of survivors of HSCT. Briefly, this multivariable model proposes that individual psychological outcomes after a major health crisis, such as undergoing HSCT, will be influenced by personal (e.g., age and gender), clinical (e.g., diagnosis, time since diagnosis and conditioning protocol) and familial factors.

Given the added medical demands for the ill child with this procedure and the great deal of attention they receive, we predicted that survivors will show better psychosocial outcomes than their siblings, but will still show deficits in cognitive and educational outcomes and physical QOL. Given our previous findings that familial factors contributed to the survivors' cognitive and educational outcomes, and that both survivors and siblings have different experiences within the same family, it is likely that, when differences exist, familial factors such as maternal depression scores will be associated with poorer outcomes in survivors. This study was part of a major project investigating the psychosocial and educational outcomes in a family after pediatric HSCT.

Materials and methods

Participants and recruitment
Potential survivor participants for this investigation were children over the age of 3 years who participated in the longitudinal study of the larger project and had complete data at 2 years post-HSCT. All the families that had eligible siblings agreed to participate in the longitudinal study but not all ill children survived 2 years post-HSCT. Complete data were obtained for 46 survivors and 33 siblings. The inclusion criteria for siblings were as follows: age within 2 years of the target child's age and presenting with no major health problems. Only one sibling per family participated and five of the siblings were donors. Comparisons related to being a donor, however, were not the focus of this investigation. Table 1 presents clinical and demographic characteristics of the survivors and siblings. Briefly, mean age of participating survivors was 10.13 years (s.d. = 4.4, range 2–16), whereas that of siblings was 10.96 years (s.d. = 3.62, range 3–20). Majority of survivors were diagnosed with other leukemia types (i.e., AML and CML), neuroblastoma and ALL. Other diagnoses included hematological disorders and Hodgkin's lymphoma. The majority of survivors (68%) underwent an autologous transplant (48% related). Seventy-four percent experienced no cranial radiation before or during conditioning. The mean time since diagnosis at pre-HSCT was 19.9 months (s.d. = 19.5). Most children came from a two-parent European-descent family and a middle-class socio-economic background. The majority of parents had undergone at least part of college or university education.

Procedure
After approval from the Research Ethics Board was obtained, an invitation letter was sent to all eligible families describing the study and inviting them to participate in the study, with the last assessment at 2 years post-transplant. Informed consent and assent were obtained at the initial assessment for the survivors. Assent or consent of siblings was obtained at their participation 2 years post-HSCT. Families were scheduled for two assessment sessions, one for the sibling and one for the survivor, to complete the cognitive, educational and visual motor performance tests and behavioral questionnaires. The mother was the primary parent during the HSCT and the one who completed the questionnaires about the survivor's and sibling's behavior and QOL, and about her own as well.

Outcome measures

General Intelligence was measured using the Wechsler Intelligence Scale for Children Third Edition (WISC-III). The Wechsler scales are well normed, producing IQ estimates labeled as verbal (VIQ), which includes information, similarities, arithmetic, vocabulary and comprehension subtests), performance (PIQ, which includes picture completion, coding, picture arrangement, block design and object assembly) and full scale (FIQ). Using the WISC-III subtests, including two supplementary subtests (digit span and symbol search), we derived specific factor-based cognitive indices: freedom from distractibility (which includes arithmetic and digit span), processing speed (which includes coding and symbol search) and perceptual organization (PO; which includes picture completion, picture arrangement, block design and object assembly) and verbal comprehension (which includes all the subtests of the verbal scale except arithmetic and digit span). The latter index was not used because it tests basically the same constructs as VIQ. Average reliability estimates for the IQ
Table 1: Clinical and familial characteristics of survivors of HSCT and their siblings

<table>
<thead>
<tr>
<th>Factor</th>
<th>Survivors (46)</th>
<th>Siblings (33)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (s.d.)</td>
<td>Mean (s.d.)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.13 (4.37)</td>
<td>10.96 (3.82)</td>
</tr>
<tr>
<td>Maternal age at HSCT</td>
<td>36.41 (5.64)</td>
<td>N % value</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>N % value</td>
</tr>
<tr>
<td>Female</td>
<td>20 43.5</td>
<td>11 32.3</td>
</tr>
<tr>
<td>Male</td>
<td>25 56.5</td>
<td>23 67.7</td>
</tr>
<tr>
<td>Diagnoses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td>8 17</td>
<td>--</td>
</tr>
<tr>
<td>Other leukemia types</td>
<td>12 26</td>
<td>--</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>14 30</td>
<td>--</td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
<td>4 9</td>
<td>--</td>
</tr>
<tr>
<td>Hematologic disorders</td>
<td>8 17</td>
<td>--</td>
</tr>
<tr>
<td>Time since diagnosis (months)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30</td>
<td>15 32.6</td>
<td>--</td>
</tr>
<tr>
<td>30-36</td>
<td>16 34.2</td>
<td>--</td>
</tr>
<tr>
<td>&gt;36</td>
<td>15 32.6</td>
<td>--</td>
</tr>
<tr>
<td>Type of transplant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allogeneic</td>
<td>38 83</td>
<td>--</td>
</tr>
<tr>
<td>Related</td>
<td>22 48</td>
<td>--</td>
</tr>
<tr>
<td>Unrelated</td>
<td>16 35</td>
<td>--</td>
</tr>
<tr>
<td>Autologous</td>
<td>8 17</td>
<td>--</td>
</tr>
<tr>
<td>Radiation history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No radiation</td>
<td>32 74</td>
<td>--</td>
</tr>
<tr>
<td>TBR only</td>
<td>10 22</td>
<td>--</td>
</tr>
<tr>
<td>CRT and TBR</td>
<td>4 9</td>
<td>--</td>
</tr>
<tr>
<td>Radiation group (cGy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;300</td>
<td>35 76</td>
<td>--</td>
</tr>
<tr>
<td>&gt;4000</td>
<td>11 24</td>
<td>--</td>
</tr>
<tr>
<td>Maternal education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Part of high school</td>
<td>3 6</td>
<td>--</td>
</tr>
<tr>
<td>Complete high school</td>
<td>8 17</td>
<td>--</td>
</tr>
<tr>
<td>Part of college/university</td>
<td>12 26</td>
<td>--</td>
</tr>
<tr>
<td>Complete college/university</td>
<td>23 50</td>
<td>--</td>
</tr>
</tbody>
</table>

Abbreviations: CRT = cranial radiation therapy; HSCT = hematopoietic SCT; TBR = total body radiation.

Measures of related factors

The Family Adaptability and Cohesion Evaluation Scale (FACES-III) and the Beck Depression Inventory (BDI) were used as the measures of family cohesion and maternal distress, respectively, as potential related factors. These measures were chosen because they are brief and widely used in families with a history of childhood chronic illness or cancer and could be completed by a parent with minimal burden. FACES-III is a 20-item scale with two dimensions of family relations: adaptation and cohesion. Test–retest reliability over a 4- to 5-week period was r = 0.80 for adaptability, and r = 0.83 for cohesion, with high discriminative validity. In this study, the cohesion score was used as a measure of family functioning. The BDI is a self-administered 21-item questionnaire designed to measure depressive symptomatology. The BDI has been found to be highly sensitive in measuring changes in depressive symptoms and severity. Concurrent validity of the BDI with most other self-report measures of depression has been consistently high. Both measures were completed by the mother.

Clinical and demographic factors

Information on the child’s disease and treatment was obtained from the medical chart and confirmed by parental report. The clinical variables considered in this study were as follows: diagnosis type (ALL, other leukemias, neuroblastoma, Hodgkin’s lymphoma and hematological disorders), time since diagnosis (stratified into less than 30, between 30 and 36, and more than 36 months), history of radiation treatment (no radiation, total body radiation (TBR) only, and combined cranial radiation therapy (CRT) and TBR) and radiation group (<300 cGy or >1200 cGy). The child characteristics included in this study were as follows: child’s age at pre-HSCT and gender. Maternal age and education were also considered, with the latter as an index of socioeconomic status.
Statistical analysis

Initially, descriptive statistics were calculated for the outcome measures and related variables. Because of the small sample size and missing data for some of the variables (see Table 2), all the outcome measures were tested for normality using the Kolmogorov–Smirnov test. The majority of the variables did not violate the assumption of normality. However, because the FIQ and VIQ scores were not normally distributed, they were tested using both parametric and non-parametric tests in the comparisons between survivors and siblings. These results were similar, hence only the parametric results are reported. Paired t-tests were conducted to compare survivor and sibling scores on all measures (cognitive, visual motor, educational, psychosocial and QOL). When there was a significant difference between survivors and siblings, we examined what factors may have accounted for the difference. Thus, the following analyses were conducted to select and reduce potential predictors of differences. To avoid collinearity, a pair-wise assessment of associations between predictors was examined. That is, χ²-tests within categorical predictors, analysis of variance for continuous and categorical and Pearson correlations for continuous variables were conducted. The variables found to have high potential collinearity were not included together in a multivariable model. Covariates significant at the 20% level in the bivariate analyses were included in the full multivariable, generalized linear regression model. History of radiation was chosen over radiation group based on these preliminary analyses. Generalized linear regression models were then constructed to examine the effects of the potential factors associated with the differences between survivors and siblings. These analyses were conducted using SAS software version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

Differences in outcome measures between survivors and siblings

Table 2 presents the means and s.d. of the outcome measures for survivors and their siblings, as well as the related factors.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Means and s.d. for outcome and related measures of survivors of HSCT and their siblings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>Survivors</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Insestion scales (IQ)</td>
<td></td>
</tr>
<tr>
<td>Full</td>
<td>46</td>
</tr>
<tr>
<td>Verbal</td>
<td>46</td>
</tr>
<tr>
<td>Performance</td>
<td>46</td>
</tr>
<tr>
<td>Cognitive indices</td>
<td></td>
</tr>
<tr>
<td>Freedom from distraction</td>
<td>46</td>
</tr>
<tr>
<td>Perceptual organization</td>
<td>46</td>
</tr>
<tr>
<td>Processing speed</td>
<td>32</td>
</tr>
<tr>
<td>Visual motor Integration</td>
<td>44</td>
</tr>
<tr>
<td>Academic (WRAT)</td>
<td></td>
</tr>
<tr>
<td>Spelling</td>
<td>41</td>
</tr>
<tr>
<td>Reading</td>
<td>42</td>
</tr>
<tr>
<td>Arithmetic</td>
<td>42</td>
</tr>
<tr>
<td>Child behavior (CBCL)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
</tr>
<tr>
<td>Internalizing</td>
<td>46</td>
</tr>
<tr>
<td>Externalizing</td>
<td>46</td>
</tr>
<tr>
<td>QOL</td>
<td></td>
</tr>
<tr>
<td>Physical</td>
<td>42</td>
</tr>
<tr>
<td>Psychosocial</td>
<td>42</td>
</tr>
<tr>
<td>Family factors</td>
<td></td>
</tr>
<tr>
<td>Maternal depression</td>
<td>46</td>
</tr>
<tr>
<td>Family cohesion</td>
<td>45</td>
</tr>
</tbody>
</table>

Abbreviations: CBCL = Child Behavior Checklist; HSCT = hematopoietic SCT; QOL = quality of life; WRAT = Wide Range Achievement Test.
*P<0.05.
**P<0.001.

Cognitive and visual motor outcomes. Mean FIQ, PIQ and VIQ scores of survivors did not differ significantly from the corresponding mean scores of their siblings. Although survivors' mean PIQ scores were higher than those of their siblings, this difference did not reach significance (t(31) = 1.70, P = 0.10). Analysis of the cognitive indices indicated that the mean for PO was significantly higher for survivors than the mean for siblings (t(31) = 2.14, P = 0.04). Both PO scores, however, were within the normal range. Moreover, the VMI mean standard scores for survivors and siblings did not differ significantly.

Educational outcomes. Siblings' mean scores for spelling were significantly higher than those of survivors (t(25) = 2.06, P < 0.05), with the siblings' scores being more than 7 points higher. The mean scores for arithmetic and reading were in the same direction but did not reach significance.

Psychological adjustment. The mean score for total CBCL, and externalizing scores, did not differ significantly between survivors and siblings but the difference between their mean internalizing scores was significant, with the siblings scoring 4 points higher than the survivors, suggesting more internalizing problems (t(32) = -2.66, P < 0.02). However, as a group, the mean internalizing score for survivors and siblings were within the normal range.

Quality of life. The mean physical QOL summary score for survivors was significantly below the mean for siblings, suggesting more physical difficulties in survivors 2 years post-HSCT (t(31) = -4.31, P < 0.0001), with a difference of 10 points. The mean psychosocial QOL summary scores did not differ significantly between the groups.

Factors associated with the differences between survivors and siblings

To further understand the differences found between the survivors and siblings in the cognitive index of PO, the
educational outcome, spelling, psychological adjustment, measured by internalizing scores, and physical QOL, next we examined potential clinical factors (diagnosis, history of radiation, time since diagnosis, type of transplant), child characteristics (age) and family factors (maternal age and depression scores, family cohesion) that might be related to these differences. Preliminary analyses indicated that none of the clinical variables were associated with the difference between siblings and survivors in PO, which reflected better scores for the survivors than the siblings. Two familial factors, maternal age and family cohesion, were identified in preliminary analyses, using parametric and nonparametric statistics, as associated with the difference between the groups in PO and included in the regression model. The model was significant ($R^2 = 0.60$, $F_{2,14} = 9.30$, $P = 0.0036$), with maternal age ($F_{1,14} = 13.79$, $P = 0.02$) and family cohesion ($F_{1,14} = 8.92$, $P = 0.011$) being associated to the difference, in spite of the small sample that had family cohesion data. These results indicate that the older the mothers, the higher the differences in favor of the survivor. Moreover, the difference was greater in more cohesive families.

For the difference in spelling, which reflected better outcomes for siblings than for survivors, the regression model ($R^2 = 0.47$, $F_{2,22} = 9.83$, $P < 0.0001$) included two factors: child age ($F_{1,22} = 8.76$, $P = 0.007$) and maternal depression ($F_{1,22} = 4.54$, $P = 0.04$), suggesting that the difference in spelling scores was smaller with older survivors and when maternal depression scores were low.

For the difference in internalizing scores, which reflected more internalizing problems for siblings than for survivors, the significant model ($R^2 = 0.51$, $F_{4,20} = 4.59$, $P = 0.0026$) included history of cranial radiation ($F_{2,20} = 3.35$, $P = 0.05$) and diagnosis ($F_{5,20} = 3.04$, $P = 0.03$). This analysis suggests that the difference was smaller if the survivors had combined CRT and TBR or a diagnosis of neuroblastoma or Hodgkin’s lymphoma. Finally, none of the variables tested (personal, clinical and familial) were associated with the difference between survivors and siblings in physical QOL, suggesting that this difference is primarily a result of disease and HSCT treatment.

Discussion

Contrary to predictions, survivors of HSCT performed as well in global and specific cognitive outcomes as their siblings but better than their siblings in PO. As predicted, they showed some deficits in educational outcomes and physical QOL. Moreover, their psychosocial QOL was rated as good as that of their siblings and, as a group, they presented with less internalizing problems than their siblings. These encouraging results support the previous longitudinal outcomes in this population, which showed improvement on PIQ and stable overall cognitive performance during the first 2 years post-HSCT, outcomes that were equivalent to or better than the normative values. It was surprising, however, that survivors were in fact doing better than their siblings on the specific index of PO, which measures specific ability to think in terms of visual images and manipulate them with fluency. This ability is associated with persistence and alertness, and perhaps reflects the survivors’ experiences during the hospitalization and recovery period. During this period, survivors usually spend a substantial amount of time playing video games, which can enhance the skills measured by the PO index.

It was not a surprising finding that two familial factors, maternal age and family cohesion, were associated with the difference in scores in favor of the survivors. This result suggests that the children experienced different environments, one that may have resulted in greater individual attention in favor of the survivors because of their medical condition. This finding, however, is limited by the small sample size. Another familial factor, maternal depression scores, was associated with the difference in spelling. Thus, if mothers had high depression scores, survivors had lower spelling scores than their siblings, suggesting that survivors’ attention and memory for writing might have been influenced by changes in maternal mood.

It was not surprising, however, to see that the difference in spelling was smaller for older groups, as older children are more likely to have learned spelling patterns before HSCT. Thus, the findings of this study align with the previous longitudinal educational outcomes suggest that a younger age at HSCT and indices of poor maternal psychological well being (measured by depression scores) may be risk factors for their educational outcomes. Consequently, these findings highlight the importance of early school attendance for learning the fine details of written language before HSCT, and identifying an area of intervention focus for schooling children during the first 2 years post-HSCT. Moreover, these findings also suggest that family support and counseling pre- and post-HSCT may result in indirect positive effects on cognitive and educational outcomes.

Although, compared with their siblings, survivors, as a group, did not present with internalizing difficulties, it was interesting to see that survivors with a history of cranial radiation or a diagnosis of neuroblastoma or Hodgkin’s lymphoma were likely to have high internalizing scores as their siblings. This finding raises the possibility that survivors with a history of cranial radiation, and those who survive neuroblastoma and Hodgkin’s lymphoma, are potentially at risk for internalizing problems. It is important to stress that neither survivors nor siblings, as a group, had mean internalizing scores in the clinical range. This attests to children’s resilience to the effects of traumatic medical experiences. Still, the fact that siblings in general tended to have more internalizing problems (anxiety, depression and social withdrawal) than survivors supports some earlier findings. Recent reports on post-traumatic stress symptoms in siblings of children with cancer, and suggests that some siblings, in fact, were reported to have internalizing scores in the clinical range.

It was not surprising to find poorer physical QOL in survivors compared with siblings, given their previous disease and aggressive treatment. It was interesting to see, however, that in spite of these physical limitations, psychosocially they were rated to be doing at least as well as their siblings. Finally, none of the additional personal...
and familial factors examined in this study were associated with the group difference in physical QOL, which suggests that this difference was directly a result of the disease and treatment, particularly, undergoing HSCT. In spite of the physical problems, however, survivors remain cognitively and emotionally resilient and continue to live, what appear to be, well-adjusted lives. The fact that our results show lesser deficits than reported previously may also reflect current less-aggressive protocol treatments, for example, reduced cranial radiation for ALL patients.

This study has several limitations. A small sample and missing data resulted in reduced statistical power and limited the contribution of this study. Having a small sample size is not unusual in research with this medically vulnerable population faced with poor health, many stressors and high mortality rates.3 Comparing the outcomes of these survivors with those of their siblings is an asset as it controls for both environmental and genetic variance. Conversely, having the mother complete reports on both survivors and siblings limits the data on questionnaires to maternal perspective. The study was also limited by the availability of siblings as not all the survivors have a sibling close in age to complete the assessment measures. Although there are suggestions that being a sibling donor may make a difference in the sibling outcomes, comparisons of sibling donors and non-donors could not be made due to the small sample size of donors (five). Future studies should compare these outcomes among these groups of siblings and survivors. Despite these limitations, the outcomes of this comprehensive comparative study are unique, as they illustrate the resilience of survivors and the impact of pediatric HSCT within the family.

In conclusion, 2 years post-HSCT survivors' PO and psychological adjustment seem to be at least as good as those of their siblings, but their physical QOL and educational outcomes showed some deficits. Child characteristics, such as age, but mainly familial factors (maternal age and depression and family cohesion) seem to be associated with the difference in cognitive and educational outcomes between survivors of HSCT and their siblings. Conversely, clinical factors—history of cranial radiation and type of diagnosis—seem to be associated with psychological adjustment differences. These findings provide new knowledge for corroboration by future researchers. Finally, these findings have important implications for comprehensive family care, particularly in light of the important positive association of the outcomes with maternal well being and age.

Acknowledgements

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References

Indeed, our study of prospective EBV monitoring was initiated because of a case similar to the one described by Wondergem et al. Our patient was a 32-year-old man with severe aplastic anemia who received horse antithymocyte globulin (ATG) with no response at 3 months and was then treated with rabbit antithymocyte globulin. Two weeks later, rapidly progressive massive lymphadenopathy developed in the neck, axillary, and mediastinal areas, requiring endotracheal intubation. Axillary lymph node biopsy revealed EBV lymphoproliferation, accompanied by an EBV viral load of 870,000 copies per 10⁶ mononuclear cells in the blood. Cyclosporine was discontinued and the patient received one cycle of cyclophosphamide, doxorubicin, vincristine, prednisone plus rituximab (CHOP-R) with a rapid decrease in node size and in peripheral blood EBV copy numbers. He went on to unrelated hematopoietic stem cell transplantation for his aplastic anemia and 4 years later he is doing well with no further evidence of EBV disease.

Notwithstanding, EBV-lymphoproliferative disease after immunosuppression for aplastic anemia is very rare and its occurrence has been limited to case reports. We have now monitored EBV reactivations in more than 150 courses of immunosuppressive therapy with no additional cases of EBV disease, despite very high viral loads and prolonged periods of EBV polymerase chain reaction (PCR) positivity. In the past 18 months we have monitored EBV viral loads in patients who received rabbit ATG as initial therapy; higher viral loads were again observed in patients treated with the rabbit ATG on top of similar levels to what was reported in the manuscript when rabbit ATG was administered as a second course only compared with those who received horse ATG. Therefore, the EBV viral load cannot be interpreted in isolation as cut-off values that are predictive or diagnostic of disease have not been established. Rather than mandate routine testing for what we believe is a rare event, we would instead prefer to stress awareness of the potential for EBV reactivation and disease, with intervention only when there is clinical suspicion due to rising lactate dehydrogenase (LDH), lymphadenopathy, clinical deterioration in association with a high EBV viral load, and confirmatory lymph-node histology.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.
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To the editor:

Physical and not mental health is impaired in very long-term survivors after HSCT compared with their respective donors: a paired analysis

Bhushan and colleagues recently published a comprehensive analysis on late mortality after allogeneic hematopoietic stem cell transplantation (HSCT), providing interesting data about the functional status of 547 recipients and 319 siblings. At the time of this Collaborative Bone Marrow Transplant Survivor Study, patients and donors had a median age of 41.5 and 44 years, respectively, and a median time of 8.6 years after HSCT. By questionnaire, long-term survivors reported significantly more difficulties in integration back into society after HSCT, in holding down employment, or in obtaining or retaining health insurance compared with their siblings. These results provided additional information to the relatively scarce and partially conflicting reports on functional status in the long-term recipients of HSCT surviving more than 10 years.2,3

In order to obtain a comprehensive overview on physical and mental health in very long-term survivors after HSCT, we invited 44 recipients and their respective HLA-identical sibling donors to take part in a prospective study at the University Hospital of Basel. Both the recipients and their donors were controlled at the same time point, in pairs, and were given a complete clinical and biologic examination. Each answered a Short Form 36 (SF-36) Health Survey, which provides the generic health status measure using 36 items assessing 8 different concepts (Table 1). Three of the concepts provide a score for physical health, 3 for mental health, and 2 for general health status. These 8 concepts are summarized in 2 global tests, one for physical and one for mental health. Norm-based scores were used, in which 50 represents the mean score, and 10 the standard deviation for the general population. The median age of the recipients and donors at the time of the study was 44.3 years (24-63) and 43.4 years (22-61), respectively, with a median time of 17.5 years (range, 11-26 years) after HSCT. Four patients received an HSCT for aplastic anemia and 40 for hematologic malignancies. All patients received bone marrow as stem cell source and total body irradiation was part of the conditioning in 39 patients (89%). Acute graft-versus-host disease (GVHD) was observed in 31 (70%), and chronic GVHD in 22 (50%) patients.

In a paired comparison, recipients showed a significantly lower rank of the norm-based scores for all questions related to physical well-being, except for role limitation, but no difference in the mental health scores compared with their respective donors (Table 1). This is confirmed by the global test for physical (P = .001) and mental (P = .831) health. Physical health was significantly lower in patients with extensive chronic GVHD (P = .05), in females (P = .024), in recipients older than 25 years at HSCT (P = .024) or older than 42 years at study evaluation (P = .05). None of these factors had a impact on mental health.

Table 1. Short Form 36 Health Survey: Paired comparison between recipients and their respective donor

<table>
<thead>
<tr>
<th>Items</th>
<th>Donor</th>
<th>Recipient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Items describing physical health</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical functioning</td>
<td>54.7</td>
<td>50.9</td>
<td>.001</td>
</tr>
<tr>
<td>Role limitations due to physical health</td>
<td>54.6</td>
<td>52.3</td>
<td>.213</td>
</tr>
<tr>
<td>Bodily pain</td>
<td>59.2</td>
<td>54.0</td>
<td>.042</td>
</tr>
<tr>
<td>Items describing general health (physical and mental)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>General health perception</td>
<td>57.0</td>
<td>50.7</td>
<td>.001</td>
</tr>
<tr>
<td>Vitality</td>
<td>65.4</td>
<td>52.9</td>
<td>.039</td>
</tr>
<tr>
<td>Items describing mental health</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Social functioning</td>
<td>54.0</td>
<td>51.2</td>
<td>.524</td>
</tr>
<tr>
<td>Role limitations due to emotional problems</td>
<td>53.9</td>
<td>51.0</td>
<td>.285</td>
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<tr>
<td>Mental health</td>
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<td>Global tests</td>
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<tr>
<td>Physical Component Summary (PCS)</td>
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<tr>
<td>Mental Component Summary (MCS)</td>
<td>52.9</td>
<td>50.8</td>
<td>.031</td>
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</table>

* Numbers are means of norm-based scores.
health status (MCS, \( P > .10 \)). In summary, our data extend the findings reported by Balzar et al.\(^{2}\) on the functional health status in long-term survivors, validating the finding that the physical health status of very long-term survivors after HSCT can be impaired, while mental health status remains preserved.

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References


To the editor:

Revised criteria for the myeloproliferative disorders: too much too soon?

We would like to raise several concerns about the updated World Health Organization diagnostic criteria for polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), as proposed by Tegfæ et al.\(^{1}\)

First, while the authors explain that the new criteria are not absolutely comprehensive, they never state the exact purpose of this revision. As a purpose of diagnostic criteria is to guide diagnosis and clinical management, the authors should demonstrate that the revised criteria are validated by data from previous studies. For example, while the discovery of the JAK2V617F mutation is of paramount importance, it has not changed our ability to discriminate between the different disorders and has not changed therapeutic recommendations, although it may in the future. Without these supporting data, these criteria produce significant ambiguity for physicians attempting to decipher their clinical relevance.

Second, the new criteria emphasize differences in bone marrow morphology among the myeloproliferative disorders. But, it appears that the vast majority of this work has been done with small groups of patients in retrospective and unblinded settings, which may facilitate biases and misinterpretations. In addition, the references supporting these morphologic criteria primarily focus on the work of one of the authors. We have concerns about the general applicability of these guidelines to centers that lack the necessary hemopathology expertise. Is there sufficient confidence that evaluation of megakaryocyte morphology and fibrosis is widely reproducible among the various observers who will be attempting to make these distinctions? This concern is shared by others.\(^{2,3}\) We also question whether morphologic assessment can reliably differentiate between primary and secondary causes of myelofibrosis, such as seen in primary pulmonary hypertension, a common cause of secondary myelofibrosis. Rather that stating that "the histologic differences among the entities outlined here are recognized by experienced hemopathologists," the authors should provide objective evidence of the validity, utility, consistency, and reproducibility of the proposed criteria.

Third, the morphologic criteria have never been applied to patients with congenital polycythemia due to Von Hippel-Lindau or EPO receptor gene mutations. In our experience with these disorders, many of these patients had their narrow morphology interpreted as consistent with PV.\(^{4,5}\)

In addition, there is a major mistake in Tefëfi et al.'s Table 1, which summarizes the 2001 criteria; contrary to what is stated in the table, dominantly inherited polycythemia due to a truncated erythropoietin receptor is always associated with a low erythropoietin level.

In summary, we feel that before using the revised criteria as a diagnostic guide, these issues need to be further evaluated in large-scale, prospective studies, such as those being undertaken by the Myeloproliferative Disorders Research Consortium.

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Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

Differential Characterization of the "Reticulum Cell" in Lymphoreticular Neoplasms

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ABSTRACT

Yam, Lung T., Tavassoli, Mehdi, and Jacobs, Peter: Differential characterization of the "reticulum cell" in lymphoreticular neoplasms. Am J Clin Pathol. 64: 171–179, 1975. The term "reticulum cell" is confusing, having been applied to the cells involved in many hematopoietic neoplasms, such as reticulum-cell sarcoma, histiocytic medullary reticulosis, leukemic reticuloendotheliosis, and monocytic or histiocytic leukemias. In histologic sections, even the cells from poorly differentiated extramedullary lesions of chloroma or myeloblastic leukemia have been called "reticulum cells." A combined morphologic and cytochemical approach has been used to study "reticulum cells" in smears and tissue sections of neoplasms involving "histiocytes" or "reticulum cells." The cytochemical markers are: chloracetate esterase for neutrophilic granulocytes; nonspecific esterase and fluoride-resistant esterase for monocytes and histiocytes (phagocytes); tartrate-resistant acid phosphatase for the reticulum cells of leukemic reticuloendotheliosis; pyronin for the lymphatic reticulum cells (germinal center cells). The morphology of these cells is very well appreciated in smears, and the locations of these marked cells in tissue sections are easily recognized. The use of cytochemical and immunochemical methods and functional studies, in addition to simple morphology, may be useful in subclassification of lymphoreticular neoplasms. (Key words: Reticulum cell; Lymphoreticular neoplasms; Malignant lymphoma; Leukemic reticuloendotheliosis; monocytic leukemia.)

THE NEOPLASTIC DISEASES of the reticuloendothelial system comprise a variety of clinical entities. Many of these diseases have distinct clinical features and different natural histories, necessitating different therapeutic approaches. Morphologic examination of tissue sections and smears is the cornerstone of diagnosis and classification of these disorders, thereby dictating the therapeutic approach and prognosis of the disease. The inadequacy of morphologic examination based on hematoxylin–eosin-stained sections or Wright–Giemsu stained smears has long been evident by virtue of the controversies and difficulties surrounding the interpretation of the cell type involved.
A noncommittal term often used by morphologists dealing with cells involved in these disorders is "reticulum cell." It has been used to indicate a poorly differentiated cell when proper identification by conventional methods has escaped the morphologist. For example, when highly undifferentiated granulocytic neoplasms manifest as a solid tumor, it is not uncommon to have an erroneous histologic diagnosis of "reticulum-cell sarcoma."12-14 In the classic "reticulum-cell sarcoma" or malignant lymphoma of the histiocytic type, the neoplastic cell involved has been considered to be a reticulum cell by some and a histiocyte by others.10,17,18 In this situation, "reticulum cell" is synonymous with "histiocyte." In acute monocytic leukemia of the Schilling type, the cells are often called "monocytes," although others have considered these cells to be histiocytes, and the disease to be histiocytic leukemia. Here, the monocytes and their precursors are considered to be histiocytes.5,6 "Histiocytic leukemia," however, has also denoted "leukemic reticuloendotheliosis."15,16 In acute monocyctic leukemia of the Naegeli type, both myelocytic and monocytic cells proliferate, and it may be very difficult to distinguish these two types of cells accurately. In other instances, the reticulum cells are thought to be related to endothelial cells, as in leukemic reticuloendotheliosis, to plasma cells (plasmacytoid reticulum cells), or to lymphocytes (lymphatic reticulum cells).18 In some malignant lymphomas, it is not uncommon to find coexistence of many large "histiocytes" and the smaller, poorly differentiated lymphocytes, rendering it difficult to differentiate between histiocytic lymphoma and lymphocytic lymphoma. These problems demonstrate that the so-called "reticulum cells" are various in nature. A more precise definition for these cells is needed.

Previous studies have demonstrated that it is possible to identify many of the cells of the hemopoietic system both on smears and in tissue sections through the use of chemical markers.2-4,7,8,10,12,15 The present study was undertaken to study several diseases involving "reticulum cells," poorly differentiated hemopoietic cells, and "histiocytes" and to establish definite criteria for cells involved in these "reticulum-cell" diseases.

Materials and Methods

Cases studied included: histiocytic lymphoma 10; poorly differentiated lymphocytic lymphoma 10; acute myelomonocytic leukemia 10; leukemic reticuloendotheliosis 10; and one case of a patient with Sjogren's syndrome and histiocytic lymphoma (immunoblastic sarcoma). Ten patients with acute granulocytic leukemia and five patients with well-differentiated lymphocytic lymphoma were also included as controls for granulocytes and lymphocytes. The diagnoses of histiocytic and lymphocytic lymphomas were made according to the histologic criteria of Rappaport.17 The diagnosis of leukemic reticuloendotheliosis was established by identifying the typical clinical features and characteristic cytologic and histologic changes.21 The diagnoses of acute monocyctic or granulocytic leukemia were based upon the clinical pictures and the presence of leukemic cells in blood and marrow.

The tissues of 56 patients with definite neoplastic involvement were studied. In the malignant lymphomas, available tissues were lymph nodes obtained by biopsy and spleens obtained by splenectomy. In leukemic reticuloendotheliosis, the blood, marrow and spleens were studied. In acute leukemias, the tissues studied were blood and marrow in every patient and autopsy material of marrow, spleen, lymph nodes and liver from five.
Cytologic materials, including smears of blood and marrow and imprints of lymph nodes and spleens, were stained with Wright–Giemsa, methyl green–pyronin, and for acid phosphatase, tartrate-resistant acid phosphatase, nonspecific esterase, chloracetate esterase, and fluoride-resistant esterase (15 mg. NaF per 10 ml. incubation medium).\textsuperscript{12,20} Smears made from lymphocyte cultures stimulated with phytohemagglutinin (PHA) were also prepared and studied in a similar fashion.

Frozen sections of spleen and lymph node tissues were prepared and were stained with hematoxylin–eosin, and for acid phosphatase, tartrate-resistant acid phosphatase, nonspecific esterase, and fluoride-resistant esterase.\textsuperscript{13} After the cryostat sections had been made, tissues were further embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin–eosin, reticulin stain, and methyl green–pyronin.

Several types of cells are defined on smears with Wright–Giemsa stain, and on paraffin-embedded sections stained with hematoxylin–eosin (Figs. 1–8). Detailed descriptions of these cells are listed in the appendix. The terminology used here is solely for the purpose of this communication. These terms have been used, or are being used, in the hematologic literature, but may be subject to evolutionary change.\textsuperscript{15}

Results

Cytometric Characteristics of the Cell Types

The cytometric characteristics of the various types of cells are listed in Table 1.

The lymphatic reticulum cells of Moeschlin (germinal center cells), immunoblasts, typical and atypical lymphocytes, and lymphoblasts all share common cytometric features. They lack chloracetate esterase activity but have weak to moderate acid phosphatase activity and minimal nonspecific esterase, fluoride-resistant esterase and tartrate-resistant acid phosphatase activities. The cytometric characteristics of these cells are similar to those of the PHA-stimulated lymphocytes.

Both histiocytes and monocytes contain strong activity of acid phosphatase and nonspecific esterase. The histiocytes have strong activity of fluoride-resistant esterase. Moreover, they are larger than the monocytes, and often contain phagocytosed material. A few histiocytes particularly the epithelioid variant, do have tartrate-resistant acid phosphatase activity. By contrast, the monocyte and its precursors do not show significant activity of fluoride-resistant esterase or tartrate-resistant acid phosphatase. The immature monocytes are moderately positive for methyl green–pyronin stain.

The immature granulocytes are characterized by having chloroacetate esterase activity but negligible activity of tartrate-resistant acid phosphatase, nonspecific esterase and fluoride-resistant esterase. They have variable pyroninophilia.

The hairy cells or neoplastic reticulum cells of leukemic reticuloendotheliosis are characterized by a strong tartrate-resistant acid phosphatase activity. They differ from the histiocytes with tartrate-resistant acid phosphatase activity by lacking significant nonspecific esterase and fluoride-resistant esterase activity.

Cell Types Involved in the "Reticulum-cell" Neoplasms

The types of cells involved in the various "reticulum-cell" neoplasms are listed in Table 2.

In malignant lymphoma of the histiocytic type (reticulum-cell sarcoma), we interpret the predominant cell type as a lymphatic reticulum cell (Figs. 1–4). Lymphoblasts, atypical lymphocytes, and
histiocytes (Figs. 3 and 4) are present in small and variable numbers, ranging from 5 to 20% in most cases. In cases with a pleomorphic histologic feature, the lymphatic reticulum cells are bizarre, varying considerably in size, nuclear contour, and cytoplasmic basophilia. In cases with a mixed histiocytic-lymphocytic histologic picture, the imprints show increased numbers of lymphoblasts and lymphocytes or histiocytes interspersed with the lymphatic reticulum cells.

In the poorly differentiated lymphocytic lymphomas, the predominant cells are the lymphoblast and the atypical lymphocyte (Fig. 6a and b), although some lymphatic reticulum cells, typical small lymphocytes, and phagocytic histiocytes are also present.

In the well-differentiated lymphocytic
Figs. 1–8. Top, 1–4; center, 5a, b, 6a, b; bottom, 7a, b, 8a, b.

Fig. 1. Paraffin-embedded section of lymph node from a patient with histiocytic lymphoma, showing the characteristic "histiocytes" (arrows) with prominent nuclei. Note that many smaller lymphocytes with pyknotic nuclei are also present. Hematoxylin and eosin, ×1,400.

Fig. 2. Lymph node imprint from the same patient as in Figure 1, showing the characteristic "histiocytes" or lymphatic reticulum cells (coarse arrows) with prominent nuclei. These cells have deep blue cytoplasm and an intense cytoplasmic stain with methyl green–pyronin. The cell in the right lower corner (fine arrow) is probably a lymphoblast, and may be identical to the cell indicated by the fine arrow in Figure 1. Wright–Giemsa, ×1,400.

Fig. 3. Paraffin-embedded section of lymph node from a patient with histiocytic lymphoma, showing histiocytes with and without phagocytosis (arrows). Hematoxylin and eosin. ×1,400.

Fig. 4. Lymph node imprint from the same patient as in Figure 3, showing one histiocyte (arrow) and several lymphatic reticulum cells. Wright–Giemsa, ×1,400.

Fig. 5. Lymph node section (5a, hematoxylin and eosin) and imprint (5b, Wright–Giemsa) from a patient with immunoblastic sarcoma, showing the typical large immunoblasts, mature plasma, and transitional forms (arrows) ×1,400.

Fig. 6. Two types of "lymphosarcoma cell": one with a huge nucleolus and prominent nucleolar membrane (6a, blood) and the other with a notched nucleus (6b, lymph node imprint). The lymphoblast simulates the cell in 6a but has a smaller nucleolus. Wright–Giemsa, ×1,400.

Fig. 7. Myeloblasts from a patient with acute granulocytic leukemia (7a) and monoblasts from a patient with monocytic leukemia (7b). Wright–Giemsa, ×1,400.

Fig. 8. Bone marrow section (8a, hematoxylin and eosin) and smear (8b, Wright–Giemsa), from a patient with leukemic reticuloendotheliosis, showing the typical hairy cells. ×1,400.

Lymphomas, typical lymphocytes are seen on smears and imprints, and small, pyknotic lymphocytes are seen on sections. Atypical lymphocytes with folded or lobulated nuclei are uncommon in these cases.

In the one case of histiocytic lymphoma associated with Sjögren’s syndrome (immunoblastic sarcoma), the predominant cells are immunoblasts and plasma cells (Fig. 6a and b). Some lymphatic reticulum cells, atypical and typical lymphocytes, and histiocytes with epithelioid features are also present.

In the acute leukemias, the granulocytes and their precursors predominate in cases of acute granulocytic leukemia, although some monocytes are also present (Fig. 7a): the monocytes and their precursors are the predominating cells in acute monocytic leukemia (Fig. 7b), but variable numbers of immature granulocytes are also present. Histiocytes with strong nonspecific esterase activity and fluoride-resistant esterase are not found in the blood smears from these patients.

In leukemic reticuloendotheliosis, hairy cells are present in the blood, marrow, spleen and lymph nodes (Fig. 8). This cell is pathognomonic of the disease.

Discussion

Morphologic examination of several cell types in the reticuloendothelial system, e.g., monocytes, immature lymphocytes, histiocytes, and stem cells, lacks definite criteria needed for accurate cell identification. This is particularly true when the cell type is relatively immature—thereby lacking even the usual morphologic characteristics. The problem is further compounded in neoplastic disorders of the reticuloendothelial system by abnor-
malities resulting from the neoplastic nature of cell proliferation. Thus, the term “reticulum cell” has been used to connote several different types of cells. The confusion in defining the “reticulum cell” has created much difficulty in dealing with the “reticulum-cell” neoplasms.

Cytochemical markers provide an additional dimension to morphologic identification. Cells of the same size, shape, and cytoplasmic-nuclear ratio, in addition to possessing similar folded nuclei, may differ in their origins and natures. Enzymatic differences indicated by cytochemistry provide clues to the exact natures of these cells. Furthermore, some cells, easily identified on a smear or imprint, may appear quite different in sections. Here, sometimes, the cytochemical marker can provide essential evidence that the two are the same.

The present study indicates that the cytochemical markers, as used herein, can be helpful in defining the cell types often considered “reticulum cells.” The monocytes predominate in acute monocytic leukemia. They are phagocytic, and are characterized by strong activity of nonspecific esterase. The histiocytes are phagocytic and are unique in having strong activity of a fluoride-resistant esterase. They are present in small numbers in tissue but have not been found in blood, even in pathologic conditions. The immature granulocytes seen predominately in acute granulocytic leukemia have strong activity of chloracetate esterase but very weak or no activity for nonspecific esterase. The hairy cells are pathognomonic for leukemic reticuloendotheliosis. They have a hairy appearance, strong tartrate-resistant acid phosphatase activity, and, usually, insignificant activity of esterases. The lymphatic reticulum cells that predominate in cases of “histiocytic lymphoma” are morphologically similar to the lymphatic reticulum cells (germinal center cell) of
Moeschlin and to the completely transformed lymphocytes stimulated by PHA. They do not have phagocytic activity. The lymphoblasts and atypical lymphocytes are seen often in poorly differentiated lymphocytic lymphomas. They have no positive cytochemical markers and may require the use of surface markers and other immunochemical methods for accurate identification and classification.

We have used the term “lymphatic reticulum cell” to describe the “reticulum cells” or “histiocytes” that are predominant in malignant lymphoma of the histiocytic variety included in this study. These cells are considered to be lymphocytic in origin because they resemble activated lymphocytes, both morphologically and cytochemically. Transitional forms between these cells and the lymphoblasts occur often. Thus, an analogy can be made between the lymphocytes of malignant lymphomas and the lymphocytes cultured in the presence of PHA. Morphologically, the lymphatic reticulum cells closely resemble the lymphocytes transformed in the presence of PHA, whereas the lymphoblasts and atypical lymphocytes seen in poorly differentiated lymphomas resemble partially transformed cells. The lymphocytes in well-differentiated lymphocytic lymphoma resemble lymphocytes that are unstimulated or poorly stimulated in the presence of PHA. In malignant lymphomas, the lymphatic reticulum cells are seen predominantly in “histiocytic” lymphoma; the lymphoblasts and the atypical lymphocytes in poorly differentiated lymphocytic lymphoma; and the typical lymphocytes in well-differentiated lymphocytic lymphoma. In lymphocyte cultures stimulated with PHA, the unstimulated or partially stimulated cells predominate at days 1 and 2, while fully transformed cells are present on day 3 and thereafter. In addition, the extent of stimulation of the lymphocytes is directly proportional to the concentration of the PHA added to the culture medium. It is possible that the malignant lymphomas, be they well-differentiated, poorly differentiated or histiocytic, represent a cellular response to a continuous stimulation by some as yet unidentified antigens; different “antigens” may result in various extents of cellular response. Thus, a milder and shorter stimulation would give rise to a less profound cellular reaction, as in the well-differentiated lymphoma; while a

| Table 2. Cell Types Involved in the Lymphoreticular Neoplasms |
| --- | --- | --- |
| Disease | Major Cell Types | Minor Cell Types |
| Malignant lymphoma, histiocytic | Lymphatic reticulum cells | Lymphoblast, atypical and typical lymphocytes, histiocytes |
| Poorly differentiated lymphocytic | Lymphoblasts, atypical lymphocytes | Lymphatic reticulum cells, typical lymphocytes |
| Well differentiated lymphocytic | Typical lymphocytes | Atypical lymphocytes |
| “Immunoblastic sarcoma” | Immunoblasts, plasma cells | Histioocytes (epithelioid cells) lymphatic reticulum cells, typical and atypical lymphocytes |
| Leukemic reticuloendotheliosis | Neoplastic reticulum cells (hairy cells) | — |
| Acute monocytic leukemia | Monocytes, monoblasts | Immature granulocytes |
| Acute granulocytic leukemia | Immature granulocytes | Monocytes |
stronger and prolonged stimulation would result in a more profound cellular reaction, as in the poorly differentiated or the "histiocytic" lymphomas.

This study indicates that a combined morphologic and cytochemical approach can improve the identification and definition of the cells of the various lymphoreticular neoplasms. With the addition of immunohistochemical methods and by the use of functional criteria for the cells, it is possible to provide objective means to identify the various types of cells, and thereby to classify with improved precision this perplexing group of "reticulum-cell" neoplasms. We then may be in a better position to investigate such problems as etiology and pathogenesis of these diseases.

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References

APPENDIX

Lymphatic Reticulum Cell (Germinal Center Cell): In smears or imprints, this cell is about 20 μ in diameter (range: 16–27 μ). Its nucleus is large (12–16 μ) and round, often with fine or moderately coarse nuclear chromatin. Most of these cells contain one or several prominent nucleoli with distinct nuclear membrane. The cytoplasm is abundant, deep blue in color, and may be vacuolated. Cytologically, this cell may be indistinguishable from the phagocytized, reticuloendothelial cells. In tissue sections, the nucleus of this cell is round or oval, and some nuclei are indented, with irregular outlines. This nuclear membrane tends to be distinct and often thick. The nucleus is vesicular, and nucleoli are promi-
Nucleoli are often surrounded by a clear zone of parachromatin, variably containing a few threads of darkly-staining chromatin. Cytoplasm may appear pale, amphophilic or basophilic, and stains strongly with methyl green-pyronin reaction. This cell is often seen as the "histiocyte" in the reactive center of the malpighian follicle of the spleen and the germinal center of the lymph node. It is recognized as the "histiocyte" in the malignant lymphoma of the histiocytic variety (Figs. 1 and 2).

Histiocyte (Phagocyte): In smears or imprints, this cell is 30μ in diameter (25–40μ). It has a relatively small nucleus and abundant cytoplasm which is pale to greyish and often contains phagocytosed material. The cytoplasm margin is indistinct. The nucleus is round, oval or lobulated, with a fine chromatin network and occasionally one or two small nucleoli. Occasionally, binucleated cells are seen. In tissue sections, the histiocytes are characterized by large amounts of pale eosinophilic or clear cytoplasm, often containing phagocytosed material. The cytoplasm appears to flow between adjacent lymphoid cells. The nucleus is often round, oval or reniform, and is larger than that of a monocyte. It has a heavily and variably folded nuclear membrane. Nucleoli are relatively numerous or prominent, and chromatin is coarse, being present in moderate amount. When the hematoxylin–eosin-stained sections are not optimally stained, it may be difficult to distinguish the lymphatic reticulum cells from the phagocytic histiocytes (Figs. 3 and 4).

Immunoblast (Plasmoblast): In smears or imprints, this cell varies considerably in size and in morphology. The typical cell is large, 7μ in diameter (range: 14–22μ). The cytoplasm is abundant and is deep blue with a purple hue. The nucleus may or may not be eccentrically located in the cell, and contains one or two large bluish nucleoli with prominent perinuclear membrane. The chromatin network is characterized by large vesicular clumping. In tissue sections, these cells are characterized by large vesicular nuclei containing prominent nucleoli which are often seen in close relation to the nuclear envelope. Cytoplasm is present in moderate amount and is basophilic, often with a purple hue (Fig. 5a and b).

Lymphoblast and Atypical Lymphocytes: In smears or imprints, the lymphoblast is about 14μ in diameter (range: 12–18μ), with a large nucleus and a narrow rim of deep blue cytoplasm. The nucleus is round, often containing one or two pale nucleoli. The chromatin network is fine. The atypical lymphocytes or "lymphosarcoma cells" are usually larger than the normal lymphocytes and have more cytoplasm. Some have a very prominent nucleolus. Others often show marked nuclear variation, and have notched or folded nuclei, with or without nucleoli. The chromatin pattern is coarse and clumped. Histologically, these cells are the poorly differentiated lymphocytes, most often seen in the poorly differentiated lymphocytic lymphomas (Fig. 6a and b).

Immature Granulocytes: These include the myeloblasts, promyelocytes and myelocytes. The morphology of these cells on smears is well known and is not described here. In tissue sections, these cells have a wide rim of cytoplasm, and moderate nuclear pleomorphism with round, oval or lobulated nuclei. Nucleoli may be seen in the more primitive forms (Fig. 7a).

Monoocyte and Monoblast: In smears, the monocyte is 12–16μ in diameter. It has a round or indented nucleus with a lacy chromatin pattern. The cytoplasm is sky blue or greyish blue, often vacuolated and occasionally containing small azurophilic granules. The monoblast has a blue rim of cytoplasm, a large nucleus and one or several prominent nucleoli. In tissue sections, the monocyte has moderate amounts of pale eosinophilic or clear cytoplasm. Its nucleus is often oval or reniform with a delicate, lace-like chromatin pattern and a uniformly thin, sharply outlined nuclear membrane. Nucleoli are generally small and inconspicuous in the mature cell, but are large and prominent in the more immature forms. Phagocytosed material is occasionally present (Fig. 7b).

Hairy Cell or Reticulum Cell of Leukemic Reticuloendotheliosis: In smears or imprints, this cell is 14μ (10–20μ) in diameter. The cytoplasm is abundant, pale blue, and the cytoplasmic borders are shaggy. The nucleus is round, oval or indented, with a fine chromatin network, inconspicuous nucleoli and very distinct nuclear membrane. In wet preparations, these cells have a characteristic shaggy appearance. In histologic sections, they have abundant pale staining on water-clear cytoplasm. Their nuclei are oval or indented with fine nuclear chromatin (Fig. 8a and b).
Rapid Communication

A Clinical Evaluation of the International Lymphoma Study Group
Classification of Non-Hodgkin's Lymphoma

By The Non-Hodgkin's Lymphoma Classification Project

The recognition of several new types of non-Hodgkin’s lymphomas (NHL) in recent years has led to proposals for changing lymphoma classifications, including a new proposal put forth by the International Lymphoma Study Group (ILSG). However, the clinical significance of the new entities and the practical utility of this new proposal have not been studied. Therefore, we performed a clinical evaluation of the ILSG classification. A cohort of 1,403 cases of NHL was organized at nine study sites around the world and consisted of consecutive patients seen between 1988 and 1990 who were previously untreated. A detailed protocol for histologic and clinical analysis was followed at each site, and immunologic characterization as to T- or B-cell phenotype was required. Five expert hematopathologists visited the sites and each classified each case using the ILSG classification. A consensus diagnosis was also reached in each case, and each expert rereviewed a 20% random sample of the cases. Clinical correlations and survival analyses were then performed. A diagnosis of NHL was confirmed in 1,379 (98.2%) of the cases. The most common lymphoma types were diffuse large B-cell lymphoma (31%) and follicular lymphoma (22%), whereas the new entities comprised 21% of the cases. Diagnostic accuracy was at least 85% for most of the major lymphoma types, and reproducibility of the diagnosis was 85%. Immunophenotyping improved the diagnostic accuracy by 10% to 45% for a number of the major types. The clinical features of the new entities were distinctive. Both the histologic types and the patient characteristics as defined by the International Prognostic Index predicted for patient survival. In conclusion, we found that the ILSG classification can be readily applied and identifies clinically distinctive types of NHL. However, for clinical application, prognostic factors as defined by the International Prognostic Index must be combined with the histologic diagnosis for appropriate diagnostic and clinical decisions.

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Because of the increasing incidence of non-Hodgkin’s lymphomas (NHL), with approximately 53,000 new cases occurring annually in the United States,1,2 the diagnosis and classification of these disorders is an increasingly important clinical issue. The classification of NHL has evolved steadily throughout the twentieth century. An early system proposed by Gall and Mallory3 used the terms giant follicular lymphoma, lymphosarcoma, and reticulum cell sarcoma, but proved too imprecise for clinical application. In the 1950s, Rappaport et al4 recognized the importance of the growth pattern in some types of NHL and used patterns in addition to cell size and shape, as the basis of a new and clinically relevant classification. In the 1970s, recognition that NHLs were tumors of the immune system and were derived from T or B cells led to the immunologically based classifications of Lukes and Collins,5 and later Lennert and associates (Kiel classification).6-9 In an attempt to unify terminology and improve the effectiveness of communication between pathologists and clinicians, the Working Formulation was proposed in 1982.10 Over the next two decades, however, the Kiel classification dominated clinical practice in Europe, whereas the Working Formulation became the main classification system used in North America.

In the last two decades, increased understanding of the immune system and the genetic abnormalities associated with NHL have led to the identification of several previously unrecognized types of lymphoma. These include mantle cell lymphoma18-20 monocytoid B-cell lymphoma,21-23 extranodal lymphoma of mucosa-associated lymphoid tissue (MALT),24-28 splenic marginal zone lymphoma,29-31 primary mediastinal large B-cell lymphoma,32-34 and a variety of T-cell lymphomas,35-38 including anaplastic large cell lymphoma.18-20 The recognition of these new and supposedly clinically relevant types of NHL has led to proposals for changing lymphoma classifications. Modifications of the existing classifications, and a new proposal by the International Lymphoma Study Group (ILSG)39 incorporating some aspects of the Kiel classification and Working Formulation, have been put forward. However, the clinical significance of the new lymphoma entities and the practical utility and clinical relevance of this new proposal needed to be tested.

The histologic diagnosis of specific subtypes of NHL is widely believed to be imprecise. Previous studies have identified high rates of diagnostic discrepancy between different pathologists (interobserver variability) and for the same pathologist (reproducibility) when reviewing the same case at different times.34-38 This inaccuracy in diagnosis has made treatment decisions difficult. In the past two decades, the widespread use of immunophenotyping has led to increased insight into the diagnosis and classification of tumors of the immune system. However, the value of immunophenotyping in the day-to-day practice of lymphoma diagnosis and clinical care has not been clearly shown.

With this background, we set out to perform a retrospective clinical evaluation of the newly proposed ILSG classification.40 Although the ILSG classification is a proposal for classifying all lymphoid neoplasms (Table 1), our study was tailored to the NHL subset of this classification.
### Table 1. International Lymphoma Study Group Classification (including provisional categories)

<table>
<thead>
<tr>
<th>B-Cell Lymphoma</th>
<th>T/NK-Cell Lymphoma</th>
<th>Others</th>
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<tbody>
<tr>
<td>Precursor B-lymphoblastic</td>
<td>Precursor T-lymphoblastic</td>
<td>Composite lymphoma (types specified)</td>
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<td>Small lymphocytic (CLL)</td>
<td>T-cell chronic lymphocytic leukemia</td>
<td>Malignant lymphoma, undifferentiated low grade</td>
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<tr>
<td>Lymphoplasmacytic</td>
<td>Large granular lymphocyte leukemia</td>
<td>Malignant lymphoma, undifferentiable high grade</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>Mycosis fungoides</td>
<td>Malignant lymphoma, undifferentiable</td>
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<td>Follicular center, follicular</td>
<td>Peripheral T-cell, unspecified</td>
<td>Hodgkin's disease</td>
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<td>Medium-sized T-cell leukemia</td>
<td>Diagnosis other than lymphoma</td>
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<td>Mixed medium and large cell</td>
<td>Case undifferentiable</td>
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<tr>
<td>Marginal zone B-cell, nodal</td>
<td>Subcutaneous panniculitic</td>
<td></td>
</tr>
<tr>
<td>Marginal zone B-cell, splenic</td>
<td>Angioimmunoblastic</td>
<td></td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>Angiocentric, nasal</td>
<td></td>
</tr>
<tr>
<td>Plasmacytoma</td>
<td>Intestinal</td>
<td></td>
</tr>
<tr>
<td>Diffuse large B-cell</td>
<td>Adult T-cell lymphoma / leukemia</td>
<td></td>
</tr>
<tr>
<td>Diffuse / medium-sized large B-cell</td>
<td>Anaplastic large cell (including null phenotype)</td>
<td></td>
</tr>
<tr>
<td>Burkitt's</td>
<td>Anaplastic large cell, Hodgkin's-like</td>
<td></td>
</tr>
<tr>
<td>High grade B-cell, Burkitt-like</td>
<td>Unclassifiable low grade</td>
<td></td>
</tr>
<tr>
<td>Unclassifiable low grade</td>
<td>Unclassifiable high grade</td>
<td></td>
</tr>
<tr>
<td>Unclassifiable high grade</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Provisional categories are indicated in italic type.

Abbreviations: CLL, chronic lymphocytic leukemia; MALT, mucosa-associated lymphoid tissue.

* Follicular lymphomas are designated as such and were graded according to the Revised European American classification.†

† Composite lymphomas consisted of two distinctly different cytologic subtypes of lymphoma.

Data from Harris et al. 1986.

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designed to only assess the ILSG classification of NHL. Specific goals of our study were the following: (1) to evaluate the ability of hematopathologists to apply the ILSG classification to a retrospective group of cases collected at sites around the world; (2) to determine the role of immunophenotyping and clinical data in the diagnosis of the various entities; (3) to determine the clinical importance of immunophenotyping; (4) to determine the intraobserver and interobserver reproducibility of diagnosis of the various entities; (5) to determine clinical correlations for the various entities, including clinical features at presentation and survival outcomes; and (6) to determine whether certain entities can be grouped for prognostic or therapeutic purposes.

### PATIENTS AND METHODS

Nine institutions in eight countries were chosen to provide up to 200 consecutive cases of previously untreated NHL that were representative of the geographic region during the time between January 1, 1988 and December 31, 1990. The first 200 cases at each site that fulfilled the following criteria were selected for the study.

In all cases, tissue biopsy samples that were adequate for diagnosis and classification were required, and all diagnostic pathology materials obtained before initial therapy, including positive bone marrow (BM) specimens, were included in the pathology review. Immunologic characterization as to B- or T-cell origin, by whatever means in use at the institution, was also required in all cases. Leukemias were excluded from the study unless a tissue biopsy, other than BM, was performed before therapy. Clinical characteristics, treatment data, and some follow-up information were also required in all cases. The nine study sites, which provided a total of 1,403 cases, are shown in Table 2.

The clinical information for each case was abstracted from the medical record by a clinician or data manager and recorded on a standardized form for direct computerized data entry. These data included coded patient and site identifiers; patient sex, ethnic origin, and date of birth; the date and site of the diagnostic biopsy; and a tabulation of nodal and extranodal sites of involvement and Ann Arbor stage at the time of initial diagnosis. Laboratory data were recorded, including the serum lactate dehydrogenase level, absolute lymphocyte count, presence of circulating lymphoma cells, presence of a monoclonal serum Ig, and a history of immunodeficiency and viral (human T-cell lymphoma virus-1 [HTLV-1], human immunodeficiency virus [HIV]) status. Also recorded were the performance status and maximum diameter of the largest tumor mass. The initial therapy and therapeutic response, details of remission, progression, or relapse, and subsequent therapies and follow-up were tabulated in each case. For this report, all cases with clinical data were included regardless of the specific therapies given. In 73 of the cases, sufficient data was not available for the clinical and survival analyses.

At each institution, the pathology slides and reports for each case were carefully reviewed by a designated site pathologist. The original stained slides and immunostains were organized for review, and additional sections, immunostains, and other studies were performed if deemed necessary by the site pathologist. The results of the immunologic studies for each case, as well as any available cytogenetic or molecular genetic data, were recorded on a standardized form for direct computerized data entry. Five expert hematopathologists then traveled as a group to each of the nine sites to review and classify

### Table 2. Number of Cases by Study Site

<table>
<thead>
<tr>
<th>Site</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omaha, NE</td>
<td>200</td>
</tr>
<tr>
<td>Vancouver, Canada</td>
<td>202</td>
</tr>
<tr>
<td>Cape Town, South Africa</td>
<td>198</td>
</tr>
<tr>
<td>London, UK</td>
<td>120</td>
</tr>
<tr>
<td>Locarno, Switzerland</td>
<td>80</td>
</tr>
<tr>
<td>Lyon, France</td>
<td>195</td>
</tr>
<tr>
<td>Würzburg/Göttingen, Germany</td>
<td>210</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>200</td>
</tr>
</tbody>
</table>
ILSG CLASSIFICATION FOR NHL

Each case in each of the three major classifications. The site visits occurred over a period of 8 months beginning in June 1995. All expert pathologists used a standard Nikon Labophot-2 microscope (Nikon, Inc., Melville, NY), including a 10X plan achromat objective (high-power field = 0.159 mm²). The diagnostic categories in each of the three classifications were used according to published criteria. More specific criteria were developed for some of the entities with Nancy L. Harris providing consultation regarding the ILSG classification. The criteria of Masa and Beart were used to grade follicular lymphomas in the ILSG classification.

At each site, the diagnostic slides were reviewed and classified independently by each expert hematopathologist. The initial classification was based on examination of the hematoxylin-eosin and Giemsa stained slides with only the following clinical information from the time of initial diagnosis: patient age and sex, site of the biopsy, and the major site of disease (ie, diagnosis 1). After recording a diagnosis in each classification, the expert was then presented with the immunophenotypic profile, along with any available cytogentic and molecular genetic data, and the immunohistology and/or flow cytometry report. After review, a second diagnosis was rendered in each classification (ie, diagnosis 2). Then, the expert was presented with all of the pretreatment and clinical information and a third diagnosis was made in each classification (ie, diagnosis 3). No previous diagnosis could be changed based on information subsequently revealed.

If a case was considered subclassifiable in any of the classifications, the expert was required to give a reason, ie, inadequate material, poor slide preparation, additional phenotyping needed, additional information needed, or other reasons. The expert was allowed to change the phenotype of a case if he interpreted the immunohistology and/or phenotype data differently than the site pathologist. For some diagnostic categories, a research protocol was also completed by the expert pathologists. All of this information was recorded on standardized forms for direct computerized data entry. Approximately 40 to 50 cases were reviewed by each pathologist each day.

In addition to the independent diagnoses rendered by each of the five expert pathologists, a consensus diagnosis was also reached in each case. A consensus was considered to have been reached if at least four of the five expert pathologists agreed on the third diagnosis (diagnosis 3) in the ILSG classification. A diagnosis of follicular lymphoma of any grade was considered an agreement, and a diagnosis of peripheral T-cell lymphoma of any type was also considered an agreement. In these latter two categories, agreement by three of the five expert pathologists with regard to the specific type was considered the consensus diagnosis. If there was no agreement with regard to the type, the case was arbitrated by D. Weisenberger based on the individual diagnoses and the research protocol. All other cases without a consensus diagnosis were jointly reviewed on a multi-headed microscope and discussed by the five expert pathologists and the site pathologist in a consensus conference at the end of each day, and an attempt was made to reach a consensus of at least four expert pathologists in each case. If additional sections, immunohistology, molecular studies, or other information was required, a diagnostic algorithm was developed by the group and the additional materials were obtained, if possible, and reviewed at a subsequent consensus conference at the site. If the additional materials could not be obtained during the site visit, the required materials and information were subsequently sent to D. Weisenberger who arbitrated the case based on the algorithm.

At the end of each site visit, after all cases had been reviewed, each expert pathologist reviewed 20% of the cases. The cases for review were randomly selected by the statistician. These cases were classified a second time by each expert, without knowledge of his initial interpretation, using all available pathology materials and pretreatment clinical information. Cases in which a consensus diagnosis had not yet been reached were excluded from the review.

Table 3. Distribution of NHL Cases by the Consensus Diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>% of Total Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse large B-cell</td>
<td>422</td>
<td>30.6</td>
</tr>
<tr>
<td>Follicular</td>
<td>304</td>
<td>22.1</td>
</tr>
<tr>
<td>Grade 1</td>
<td>131</td>
<td>9.5</td>
</tr>
<tr>
<td>Grade 2</td>
<td>85</td>
<td>6.2</td>
</tr>
<tr>
<td>Grade 3</td>
<td>88</td>
<td>6.4</td>
</tr>
<tr>
<td>Marginal zone B-cell, MALT</td>
<td>105</td>
<td>7.6</td>
</tr>
<tr>
<td>Peripheral T-cell</td>
<td>96</td>
<td>7.0</td>
</tr>
<tr>
<td>Medium-sized, mixed, and</td>
<td>53</td>
<td>3.7</td>
</tr>
<tr>
<td>large</td>
<td>19</td>
<td>1.4</td>
</tr>
<tr>
<td>Angioimmunoblastic</td>
<td>17</td>
<td>1.2</td>
</tr>
<tr>
<td>Intestinal</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lymphoepithelioid</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Hepatosplenic</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Adult T-cell leukemia/lymphoma</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Small B-lymphocytic (CLL)</td>
<td>53</td>
<td>0.7</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>83</td>
<td>6.0</td>
</tr>
<tr>
<td>Primary mediastinal large B-cell</td>
<td>33</td>
<td>2.4</td>
</tr>
<tr>
<td>Anaplastic large T-cell</td>
<td>23</td>
<td>2.4</td>
</tr>
<tr>
<td>High grade B-cell, Burkitt-like</td>
<td>29</td>
<td>2.1</td>
</tr>
<tr>
<td>Marginal zone B-cell, nodal</td>
<td>25</td>
<td>1.8</td>
</tr>
<tr>
<td>Precursor T-lymphoblastic</td>
<td>23</td>
<td>1.7</td>
</tr>
<tr>
<td>Lymphomas/lymphoictoid</td>
<td>16</td>
<td>1.2</td>
</tr>
<tr>
<td>Marginal zone B-cell, splenic</td>
<td>11</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mycosis fungoides</td>
<td>11</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Burkitt's</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>All other types</td>
<td>84</td>
<td>6.1</td>
</tr>
</tbody>
</table>

Abbreviation: CLL, chronic lymphocytic leukemia.

Completed clinical and pathology forms were reviewed and edited to detect any inconsistencies, and additional information and/or classification was obtained when needed. After completion of the editing, the clinical and pathology data forms were entered into a computer for data analysis. The International Prognostic Index was used to stratify patients within the various disease entities. Treatment outcome was measured using failure-free survival and overall survival. Failure-free survival was defined as the time from diagnosis to the first occurrence of progression, relapse after response or death from any cause. Follow-up of patients not experiencing one of these events was censored at their date of last contact. Overall survival was measured from diagnosis to death from any cause, with surviving patient follow-up censored at the last contact date. Estimates of failure-free survival and overall survival distribution were calculated using the method of Kaplan and Meier. Time to event distributions were compared using the log-rank test.

RESULTS

Twenty-five of the 1,403 cases (1.8%) were found to have a diagnosis other than NHL and, thus, were excluded from further analysis. The types of NHL found in the remaining 1,378 cases are presented in Table 3. Approximately 31% of the cases were forms of diffuse large B-cell lymphoma and approximately 22% of the cases were types of follicular lymphoma. All types of T-cell processes, including natural killer (NK) cell disorders, made up only 12% of the cases. Small lymphocytic lymphoma was observed in 6.7% of the cases, a higher percentage than is sometimes appreciated.

The major newly recognized types of lymphoma that occurred most frequently were marginal zone B-cell lymphoma of MALT type (7.6%), mantle cell lymphoma (6.0%), pri-
large B-cell, and the T-cell lymphomas, immunophenotyping was helpful in many cases in reaching the correct diagnosis and improved the diagnostic accuracy by some 10% to 45%. For many of these cases, the initial diagnosis based on histology only was unclassifiable malignant lymphoma. Immunophenotyping allowed the classification of such cases into specific categories. Detailed clinical data was helpful in distinguishing primary mediastinal large B-cell lymphoma from the other diffuse large B-cell lymphomas, because there were no characteristic histologic or immunologic differences between these two categories.

The expert pathologists' review of a 20% sample of the cases at each site showed that they could reproducibly make a diagnosis of NHL (Table 5). Overall, the review diagnoses agreed exactly with the pathologist's initial diagnosis 3 or the consensus diagnosis (including the grading of follicular lymphoma) in 85% of the cases (range, 82% to 89%). Because the consensus diagnosis for all of these cases was reached before the time of the review, the consensus process may have influenced the assessment of some cases at review. Therefore, the pathologists were allowed to agree with either their original diagnosis 3 or the consensus diagnosis at the time of review. For an additional 9% of the cases, the review diagnosis was nearly the same as the original diagnosis (ie, follicular, grade 1, v follicular, grade 2; or, follicular, grade 3, v follicular, grade 3, plus diffuse large B-cell). Thus, for 94% of the cases reviewed (range, 92% to 97%), the expert pathologists made a diagnosis consistent with either their original diagnosis 3 or the consensus diagnosis. In only 6% of the cases (range, 3% to 8%) the pathologist's review diagnosis would likely have led to a different approach to therapy than the original diagnosis.

The clinical characteristics of the more common types of lymphomas are presented in Table 6. It is important to recognize that, although the average results vary between the various types, there was considerable overlap between the types for any particular characteristic. The newly recognized types of lymphoma appear to be distinctive. Marginal zone B-cell lymphomas of MALT type was characterized by a high frequency of localized extranodal disease and a prolonged survival, whereas nodal marginal zone (monocytoid) B-cell lymphoma more often presented with advanced-stage disease and had a worse survival. Mantle cell lymphoma had high grade B-cell, Burkitt-like.

### Table 4. Expert Pathologist Agreement With the Consensus Diagnosis

<table>
<thead>
<tr>
<th>Consensus Diagnosis</th>
<th>Dx 1 (%)</th>
<th>Δ Dx 2-3 (%)</th>
<th>Dx 2 (%)</th>
<th>Δ Dx 3-4 (%)</th>
<th>Dx 3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular, any grade</td>
<td>90</td>
<td>1</td>
<td>94</td>
<td>0</td>
<td>84</td>
</tr>
<tr>
<td>Follicular, grade 1</td>
<td>72</td>
<td>8</td>
<td>77</td>
<td>0</td>
<td>73</td>
</tr>
<tr>
<td>Follicular, grade 2</td>
<td>61</td>
<td>0</td>
<td>61</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Follicular, grade 3</td>
<td>60</td>
<td>1</td>
<td>61</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Marginal zone B-cell, MALT</td>
<td>84</td>
<td>2</td>
<td>86</td>
<td>0</td>
<td>86</td>
</tr>
<tr>
<td>Small lymphocytic (CLL)</td>
<td>84</td>
<td>4</td>
<td>88</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>Lymphoplasmacytoid</td>
<td>53</td>
<td>3</td>
<td>56</td>
<td>0</td>
<td>56</td>
</tr>
<tr>
<td>Primary mediastinal large B-cell</td>
<td>51</td>
<td>7</td>
<td>58</td>
<td>37</td>
<td>85</td>
</tr>
<tr>
<td>Marginal zone B-cell, nodal</td>
<td>85</td>
<td>8</td>
<td>93</td>
<td>0</td>
<td>83</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>71</td>
<td>10</td>
<td>81</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>Diffuse large B-cell</td>
<td>73</td>
<td>14</td>
<td>87</td>
<td>0</td>
<td>87</td>
</tr>
<tr>
<td>Precursor T-lymphoblastic</td>
<td>52</td>
<td>35</td>
<td>87</td>
<td>2</td>
<td>89</td>
</tr>
<tr>
<td>Anaplastic large T/nulli-cell</td>
<td>46</td>
<td>39</td>
<td>85</td>
<td>0</td>
<td>85</td>
</tr>
<tr>
<td>Peripheral T-cell, all types</td>
<td>41</td>
<td>45</td>
<td>86</td>
<td>0</td>
<td>86</td>
</tr>
</tbody>
</table>

Abbreviation: CLL, chronic lymphocytic leukemia.

* Diagnosis 1 based only on histology.
† Diagnosis 2 based on histology and immunophenotype.
‡ Diagnosis 3 based on histology, immunophenotype and clinical data.

### Table 5. Pathologist Agreement Upon Rereview of 20% of the Cases

<table>
<thead>
<tr>
<th>Cases</th>
<th>Dx 2/Consensus* (%)</th>
<th>Not-cons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall agreement</td>
<td>85</td>
<td>94</td>
</tr>
<tr>
<td>Expert pathologist</td>
<td></td>
<td>94</td>
</tr>
</tbody>
</table>

- A
- B
- C
- D
- E

* Agreement with either diagnosis 3 or the consensus diagnosis.
† Agreement including near-miss diagnoses (see text for explanation).
a striking male predominance, a high frequency of advanced-stage disease with marrow involvement, and the lowest 5-year survival of any type of lymphoma. Primary mediastinal large B-cell lymphoma occurred more frequently in young females and was often of low stage, but the survival was no different from that of other diffuse large B-cell lymphomas. Anaplastic large T/null-cell lymphoma occurred mainly in young patients and had a surprisingly high 5-year survival when compared to other lymphomas with large cell histology or a T-cell phenotype. This was not due to inactivation of a high proportion of patients with only skin involvement, a group that represented just 6% of these patients.

The average overall survival by histologic type allowed for division of the NHLs into four broad groupings (Fig 1). Those with a 5-year overall survival of greater than 70% included follicular lymphoma, marginal zone B-cell lymphoma of MALT type, and anaplastic large T/null-cell lymphoma. Lymphomas within a 50% to 70% 5-year overall survival included small lymphocytic, lymphoplasmacytoid, and nodal marginal zone B-cell lymphomas. Lymphomas with a 30% to 40% 5-year overall survival included diffuse large B-cell lymphoma, primary mediastinal large B-cell lymphoma, and the high-grade B-cell Burkitt-like and Burkitt lymphomas. Lymphomas with less than a 30% 5-year overall survival included peripheral T-cell lymphoma, precursor T-lymphoblastic lymphoma, and mantle cell lymphoma.

Whereas the histologic diagnosis of a specific type of lymphoma provides clinically important information, equally important prognostic information was obtained from the clinical characteristics of the individual patients. We found considerable variation within any particular histologic type for both overall survival and failure-free survival based on patient clinical characteristics using the International Prognostic Index (Table 7). For example, patients with follicular lymphoma had significantly different outcomes depending on their clinical prognostic characteristics (Fig 2). Moreover, patients with follicular lymphoma with a high (unfavorable) prognostic index had a far worse overall and failure-free survival (ie, 17% and 6%) than patients with a diffuse large B-cell lymphoma and a low (favorable) prognostic index (ie, 73% and 63%). In contrast, the histologic diagnosis of anaplastic large cell lymphoma was important because it was associated with a surprisingly good survival, even with a high prognostic index. In contrast, patients with mantle cell lymphoma had a relatively poor outcome despite apparently good clinical characteristics. The prognostic index also did not predict survival in precursor T-lymphoblastic lymphoma, although the number of cases was small.

**DISCUSSION**

This study shows that, using the definitions proposed in the ILSG classification, it is possible to accurately identify most of the major types of NHL. The major types recognized by this classification are also clinically distinctive, with the possible exception of high-grade B-cell Burkitt-like lymphoma, which appears to be very similar clinically to diffuse large B-cell lymphoma (Table 6). This classification was, in general, easily and accurately applied by the expert hematopathologists. In fact, this study suggests that when expert pathologists work from clear definitions, with the use of immunologic markers, the diagnosis of NHL can be made more accurately than had been thought. Previous studies, using morphology only, found that the diagnostic accuracy of specific types of NHL could not be made accurately 50% to 60% of the time. In contrast, we have shown that, when expert pathologists work from clear and agreed upon criteria, the diagnosis of NHL can be at least 85% accurate for most of the common types. However, the methods used to reach a consensus diagnosis in our study certainly had a positive influence on these agreement rates. Because treatment depends on the diagnosis, it must be made as accurately as possible. We believe that the diagnosis of NHL should always be made by a hematopathologist who is experienced in lymphoma classification.

Immunophenotyping added significantly to the accuracy of diagnosis of many of the lymphoma types, including mantle cell lymphoma, diffuse large B-cell lymphoma, and the T-cell lymphomas. However, immunophenotyping did not add significantly to the accuracy of diagnosis of some lymphomas, such as follicular lymphoma, small lymphocytic lymphoma, and marginal zone B-cell lymphoma of MALT...
Fig 1. NHLs with a 5-year overall survival of greater than 70% (A), 50% to 70% (B), 30% to 49% (C), and less than 30% (D); ALCCL, anaplastic large T/null-cell lymphoma; MZ, MALT, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue; FL, follicular lymphoma; MZ, nodal, marginal zone B-cell lymphoma of nodal type; LP, lymphoplasmacytoid lymphoma; SL, small lymphocytic lymphoma; Med LBC, primary mediastinal large B-cell lymphoma; DLCL, diffuse large B-cell lymphoma; BCL, Burkitt-like lymphoma; T-LB, precursor T-lymphoblastic lymphoma; PTCL, peripheral T-cell lymphoma; MC, mantle cell lymphoma.

| Table 7: Survival by Histologic Type and the International Prognostic Index |
|-----------------------------------------------|-----------------|-----------------|
| Consensus Diagnosis                        | 5-yr OAS Index | 5-yr FFS Index |
|                                             | 60%  | 40%  | 40%  | 40%  |
| Follicular, all grades                      | 84   | 75   | 55   | 6    |
| Mantle cell                                 | 57   | 60   | 23   | 0    |
| Marginal zone B-cell, MALT                  | 89   | 40   | 83   | 0    |
| Marginal zone B-cell, nodal                 | 78   | 50   | 30   | 0    |
| Small lymphocytic (CLL)                     | 76   | 38   | 35   | 13   |
| Diffuse large B-cell                        | 73   | 22   | 63   | 19   |
| Primary mediastinal large B-cell            | 77   | 0    | 93   | 0    |
| High grade B-cell, Burkitt-like             | 71   | 0    | 71   | 0    |
| Precursor T-lymphoblastic                   | 29   | 40   | 29   | 40   |
| Peripheral T-cell, all types                | 36   | 15   | 27   | 10   |
| Anaplastic large T/null-cell                | 81   | 83   | 49   | 83   |

Abbreviations: PI, International Prognostic Index; OAS, overall survival; FFS, failure-free survival; CLL, chronic lymphocytic leukemia.

The table shows the survival rates for different histologic types of NHLs, grouped by the International Prognostic Index. Each type, all of which have very distinctive histologic features which usually facilitate the diagnosis without a need for immunologic data. For other types, such as the lymphoplasmacytoid, nodal marginal zone B-cell, and high-grade B-cell Burkitt-like lymphomas, imprecise histologic criteria and the lack of specific immunologic markers led to a diagnostic accuracy of only 53% to 65%. Further definition of these entities is clearly needed. Because the need for immunophenotyping cannot be predicted before biopsy, it is vital that each patient have tissue available for immunophenotyping and other special studies to facilitate proper patient care. In many cases, this will require communication between the oncologist, the surgeon, and the pathologist.

The 13 major types of NHL shown in Fig 1 made up over 90% of the cases in our study, with diffuse large B-cell lymphoma and follicular lymphoma comprising over 50% of the cases and the newly recognized entities comprising 21% of the cases (Table 3). The clinical features of the various lymphoma types were remarkably different, as were
the "poor prognosis" entities. Therefore, to make proper clinical decisions, it is necessary to consider both the histologic type and the various prognostic factors present in an individual patient. Any useful clinical grouping of the NHLs must take both types of information into account.

Although the ILSG classification could be accurately applied and appears to be useful clinically, there are a number of areas that could be improved. Changes in organization and terminology have been suggested by others. In addition, more specific criteria for some of the lymphoma types are clearly needed, such as the lymphoplasmacytoid, nodal marginal zone B-cell, and high-grade B-cell Burkitt-like lymphomas. The cellular origin of so-called "marginal zone" lymphomas, along with diagnostic criteria, also need to be elucidated. Subtyping of the diffuse large B-cell lymphomas into immunoblastic and nonimmunoblastic types may be useful, and the clinical and pathologic features of anaclastic large B-cell lymphomas need to be more carefully studied before combining it into the generic category of diffuse large B-cell lymphoma. Precise criteria for grading within the various lymphoma types are clearly needed, such as for the follicular lymphomas, mantle cell lymphomas, and marginal zone B-cell lymphomas of MALT type. Finally, the number of categories of peripheral T-cell lymphomas, for which the diagnostic criteria are imprecise and difficult to apply, seems excessive when there is little evidence to support subdividing for clinical purposes. Hopefully, these issues will be addressed by the working groups of the new World Health Organization classification project.

In conclusion, the ILSG classification was readily applied and identified clinically distinctive types of NHL. Immunophenotyping added significantly to the accuracy of diagnosis in certain major lymphoma types and was clinically important. For clinical application, however, prognostic factors as defined by the International Prognostic Index must be combined with the histologic classification for appropriate clinical decisions.

ACKNOWLEDGMENT

The study participants thank Dr. Saul A. Rosenberg for his advice regarding the study design and analysis.

APPENDIX

Study Participants: The pathologists and clinicians at each institution were, respectively, Wing C. Chan and James O. Armitage (Omaha, NE), Randy Gascoyne and Joseph Conners (Vancouver, Canada), Pauline Close and Peter Jacobs (Cape Town, South Africa), Andrew Norton and T. Andrew Lister (London, UK), Ennio Pedrinis and Franco Cavalli (Locarno, Switzerland), Françoise Berger and Bertrand Coller (Lyons, France), Faith Ho and Raymond Lian (Hong Kong), Germain Ott/Alfred Schaer and Wolfgang Hiddemann (Würzburg/Göttingen, Germany). The five visiting expert hematopathologists were Jacques Diebold (Paris, France), Kenneth A. MacLennan (Leeds, UK), H. Konrad Müller-Hermelink (Würzburg, Germany), Bharat N. Nathwani (Los Angeles, CA), and Dennis D. Weisenburger (Omaha, NE). Nancy L. Harris (Boston, MA) participated as a consultant regarding application of the International
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LYMPHOMA — HISTOPATHOLOGY IN CHANGING CLINICAL PERSPECTIVE

Peter Jacobs
On behalf of the Non-Hodgkin's Lymphoma Classification Project

Background. Lymphoma management has traditionally been dominated by nodal histopathology. Unfortunately, many different classifications coexisted and frequent revisions have often obscured clinical correlations. Some improvement in understanding histogenesis followed the introduction of immunophenotyping, while a number of new entities have been described in the last decade. In addition, the whole question of lymphomagenesis is undergoing critical exploration. The use of cellular and molecular biological techniques is therefore shifting focus to the role of oncoproteins and the impact of mutation in the normally modulating suppressor genes.

To accommodate these advances the International Lymphoma Study Group has proposed the Revised European-American Lymphoma Classification. While this is an undoubted advance, it has met with persistent concerns regarding applicability to patient management.

Study setting. In determining the extent to which the latter reservation is valid, and at the same time directly testing the clinicopathological value of the new system, a group of acknowledged experts drawn from nine major academic centres worldwide analysed approximately 1 400 previously unreported cases, focusing on outcome. As part of that study 196 consecutive patients seen in Cape Town were separately examined.

Results. Findings here were similar to those of the overall experience, although distinct geographical differences emerged. Specifically in the follicular centre-cell lymphomas there was no difference in the 5-year failure-free survival rate, but these neoplasms accounted for 33% of lymphomas, which is similar to North America and London but contrasts with the 14% in the remaining six sites. Also, while mean survival for all types of peripheral T-cell lymphoma was 18% at 5 years, these accounted for 8% of lymphomas locally, as seen...
also in London and Hong Kong, but exceeding the 3-6% reported elsewhere.

Local experience, as in the other eight centres, documented good diagnostic concordance between trained haematopathologists when this classification was used by them all. Furthermore, unusual subtypes were generally well accommodated within this revised system. It should be noted that whilst histopathological features retain predictive value, they should not be considered the predominant factor. It was concluded that for management decisions to be appropriate, renewed and correct weighting must be assigned to other prognostic variables that include clinical features and markers of tumour biology.

Summary. This more enlightened prerequisite is the central goal that underlies optimal treatment outcome, since it determines stratification to appropriate and peer-reviewed protocols. It follows that review of histopathology needs to precede management of all newly diagnosed cases, preferably only by accredited multidisciplinary clinics. The previous anachronism of basking therapy on opinions of non-specialist pathologists, without appropriate review, is unwise. Furthermore, treatment by lone practitioners, or even single-specialty groups that lack the discipline to analyse their findings critically and regularly report their updated results, can no longer be considered standard of practice and should be discouraged. 

Historically, nodal architecture was used to divide these tumours into follicular lymphomas, lymphosarcomas, and reticulum-cell sarcomas. Unfortunately, this grouping was too vague to have predictive value with regard to outcome. Subsequently cytomorphology was combined with disturbances in architecture, seeking to give clinical relevance to the growth pattern. Much of that focus was changed when it was appreciated that these neoplasms originated from cells of the immune system. There followed a period of enormous confusion, since as many as 20 different classifications existed concurrently, precluding uniformity of patient entry into clinical trials, or even exchange of information between centres. Some semblance of organisation was imposed on this chaos by publication of the working formulation in 1982. Although intended as a means of interpreting data between systems, it became accepted as a classification in North America. In contrast, the Europeans favoured the system described by Karl Lennert.

This unsatisfactory state of affairs has been reassessed recently for a number of reasons. There was a growing appreciation that new and distinct variants existed. Immunophenotyping became more readily available, so that B- or T-cell origin could be determined routinely and impact of lineage could be explored as a separate variable. Cytogenetics, as in the leukaemias, was providing relevant prediction with regard to response to therapy and outcome, but was poorly accommodated in most routine reports. Furthermore, the shortcomings of older classifications were giving way to the more clinically based prognostic index.

In an attempt to redress persisting concerns, the International Lymphoma Study Group combined salient features from the working formulation with the Kiel approach to produce what is known as the Revised European-American Lymphoma (REAL) Classification. Despite this undoubted advance, some reservations have persisted, particularly among clinicians. Reservations have focused mainly on the undue weight given to histopathological features, the lack of reproducibility of diagnostic criteria between centres, or indeed even by the same pathologist, and the role that immunophenotyping should play in a practical approach to classifying, understanding and treating these neoplasms.

For these reasons an international multicentre study was undertaken to examine this new proposal. The objective was to assess its acceptability and value to practising pathologists, to explore any potential geographical differences that may exist, and specifically to determine whether it had clinical relevance. Accordingly, a collaborative effort was undertaken by clinical experts in nine areas throughout the world (Table I) working with local haematopathologists to evaluate the utility of the REAL Classification in contemporary management of patients with these lymphoproliferative disorders. In addition to publication of the global results, we now separately report the Cape Town experience.
Table I. Participants in the Multicentre International Lymphoma Study Group

<table>
<thead>
<tr>
<th>Site</th>
<th>Investigator</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Omaha, Nebraska, USA</td>
<td>Wing C Chan</td>
<td>200</td>
</tr>
<tr>
<td>Vancouver, Canada</td>
<td>Randy Gascogne</td>
<td>222</td>
</tr>
<tr>
<td>Cape Town, South Africa</td>
<td>Peter Jacobs</td>
<td>196</td>
</tr>
<tr>
<td>London, England</td>
<td>Andrew Norton</td>
<td>120</td>
</tr>
<tr>
<td>Locarno, Switzerland</td>
<td>T Andrew Lister</td>
<td></td>
</tr>
<tr>
<td>Lyon, France</td>
<td>Ennio Pedriuls</td>
<td>80</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>Francoise Berger</td>
<td>195</td>
</tr>
<tr>
<td>Würzburg/Göttingen, Germany</td>
<td>Alfred Schauer</td>
<td>200</td>
</tr>
</tbody>
</table>

Consultants were Saul Rosenberg at Palo Alto for study design and analysis, Nancy L. Harris for advice regarding the REAL Classification, James R. Anderson and Pascal Ray were statisticians, and the visiting pathologists were Jacques Diebold, Kenneth A. McMullan, H. Konrad Muller-Hermelink, Bharat Radwani and Dennis D. Weisenburger.

MATERIALS AND METHODS

Consecutive patients registered at Groote Schuur Hospital between 1 January 1988 and 1 December 1990 were identified from the records of the lymphoma clinic. The completeness of the database was confirmed by reference to the hospital central statistics department. Biopsy material, including referred slides and blocks registered in the Department of Anatomical Pathology at the University of Cape Town, was similarly scrutinised. Four hundred and twenty-six cases were identified, and after extracting pertinent clinical information, each was reviewed. Where treatment details or follow-up information was insufficient this was noted, but the case was excluded from further consideration. The lymph node and bone marrow biopsies of the remaining cases were reviewed and additional immunophenotyping was carried out where necessary. One hundred and ninety-six cases fully met the criteria for analysis (Table II). Criteria were that the tissue samples should be adequate and that all the relevant pathology materials should be available, including bone marrow aspiration and trephine biopsy samples, with immunological information being sufficient to assign the neoplasm confidently to B- or T-cell lineage. Patient characteristics, treatment data and follow-up information needed to be complete. Leukaemias were excluded.

The clinical material was compiled from review of the medical records, while histopathological preparations were reviewed by the designated site pathologist. Where necessary, additional sections were prepared and immunostains included. Other studies were performed in order that the material could be classified appropriately. Cytogenetic and molecular biological data, where available, were recorded on a standard data-capture sheet. Once compilation was complete five expert haematopathologists travelled to each of the nine centres over an 8-month period, beginning in June 1995, and spent a week reviewing all the collated information at each participating institution.

A standardised approach was used in which each of the experts first used haematoxylin and eosin- or Giemsa-stained sections to record a diagnosis when supplied only with patient age, sex, major site of disease and origin of the biopsy. The same exercise was then repeated, adding immunophenotyping together with any other cellular or molecular biological data. Finally, all available preclinical treatment information was integrated to establish a final diagnosis. At the end of each daily working session individual opinions were reviewed, with consensus defined as agreement between four of the five experts. When the site visit was complete, 20% of all the cases were randomly selected for re-review without reference to the original diagnosis.

Treatment outcomes were measured by overall and failure-free survival, with the latter defined as the time from diagnosis to first occurrence of progression, relapse after response, or

<table>
<thead>
<tr>
<th>Clinical information</th>
<th>Laboratory data</th>
<th>Follow-up measurements</th>
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<tbody>
<tr>
<td>Patient identification</td>
<td>Serum lactate dehydrogenase</td>
<td>Initial treatment regimen</td>
</tr>
<tr>
<td>Sex</td>
<td>Absolute lymphocyte count</td>
<td>Therapeutic response</td>
</tr>
<tr>
<td>Ethnic origin</td>
<td>Presence of circulating lymphoma cells</td>
<td>Details of remission, progression or relapse</td>
</tr>
<tr>
<td>Date of birth</td>
<td>Monoclonal serum immunoglobulin</td>
<td>Salvage therapies</td>
</tr>
<tr>
<td>Date and site of diagnostic biopsy</td>
<td>History of viral infections</td>
<td></td>
</tr>
<tr>
<td>Nodal areas involved</td>
<td>HIV status</td>
<td></td>
</tr>
<tr>
<td>Maximum diameter of largest tumour mass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ann Arbor staging at diagnosis</td>
<td></td>
<td></td>
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<tr>
<td>Performance status</td>
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death from any cause. Follow-up of patients not experiencing one of these events was censored at the time of last contact. Estimates of survival were calculated using the method of Kaplan and Meier and time-to-event distributions were compared using the log-rank test.

RESULTS

In the multicentre study 1.8% of cases were excluded from analysis because the diagnosis was not that of lymphoma. No such errors were recognised in the Cape Town material.

Of the consecutive locally entered patients, 33% had follicular lymphomas, 29% had diffuse large B-cell lymphomas, 8% had peripheral T-cell lymphomas, and a further 8% had small lymphocytic lymphomas. The marginal variant of the mucosal-associated lymphoid tumours was 6%, while for the mediastinal diffuse large B-cell and anaplastic large B-cell lymphomas it was 5% each, and for the mantle-cell tumours it was 1%. Twelve per cent of patients had other less frequently encountered forms of lymphoid malignancy (Table III).

As in the parent study, diagnosis based only on histology was not significantly altered by inclusion of immunophenotype when the lymphomas were follicular. These stains improved diagnostic accuracy in three other subtypes. Consequently a further 14% of diffuse large B-cell cases and an additional 39% of anaplastic large-cell cases were identified, while in peripheral T-cell variants a 45% improved recognition was possible. It should be noted that when all additional clinical data were added this was only helpful in primary mediastinal diffuse large B-cell cases. Here diagnostic accuracy improved by 37%.

It is noteworthy that when 20% of the cases were re-reviewed, the original diagnosis was verified in 80-90% of cases, suggesting that REAL Classification is reproducible in experienced hands. It is unlikely that such inter- or intra-observer reproducibility will apply to the individual who is not specifically committed to the in-depth study of these tumours.

The overall and failure-free survival rates for the Cape Town groups were 48% and 36%, respectively (Fig. 1). For patients with follicular lymphomas the corresponding figures differ by grade, both for overall survival (Fig. 2) and for those free of relapse at 7 years (Fig. 3). For the diffuse large-cell, chronic lymphocytic and peripheral T-cell lymphoma patients, overall and failure-free survival are approximately the same and can be represented by one set of graphs (Fig. 4).

DISCUSSION

Results from this local study parallel experience from other centres in supporting use of the REAL Classification on the grounds that it is currently the most appropriate approach in identifying the major types of non-Hodgkin's lymphoma. Retrospectively it became clear that in 85% of the cases examined histopathology alone sufficed for reliable diagnosis;

Table III. Clinical characteristics and laboratory data

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</thead>
<tbody>
<tr>
<td>Frequency (%)</td>
<td>33</td>
<td>28</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Median age (yrs)</td>
<td>59</td>
<td>64</td>
<td>61</td>
<td>65</td>
<td>61</td>
<td>58</td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td>Male (%)</td>
<td>42</td>
<td>55</td>
<td>56</td>
<td>53</td>
<td>45</td>
<td>41</td>
<td>34</td>
<td>69</td>
</tr>
<tr>
<td>Stage I or 2 (%)</td>
<td>33</td>
<td>51</td>
<td>18</td>
<td>6</td>
<td>66</td>
<td>18</td>
<td>66</td>
<td>50</td>
</tr>
<tr>
<td>Positive marrow (%)</td>
<td>42</td>
<td>17</td>
<td>37</td>
<td>73</td>
<td>14</td>
<td>41</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>IPFI score (%)†</td>
<td>39</td>
<td>31</td>
<td>14</td>
<td>23</td>
<td>38</td>
<td>36</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>Cytogenetics</td>
<td>t(14;18)</td>
<td>t(14;18)(q32;q21)</td>
<td>Variable</td>
<td>del(13q), +12</td>
<td>Variable</td>
<td>t(12;5)(q33;q35)</td>
<td>i(11q14)</td>
<td>(q13)</td>
</tr>
<tr>
<td>Oncogenes</td>
<td>BCL-2</td>
<td>BCL-2</td>
<td>Unknown</td>
<td>Unknown</td>
<td>+3, +13</td>
<td>ALK</td>
<td>BCL-2(Prad1)</td>
<td>BCL-6</td>
</tr>
</tbody>
</table>

While immunophenotyping was a requirement for entry, not all patients had cytogenetics performed; for completeness the data from the overall study are cited. The remaining 12% were other less frequently encountered forms of lymphoid malignancy.

* Marginal zone lymphoma: A = MALT type, B = nodal type.
† IPFI = International Prognostic Index.
this was particularly true in those cases with a follicular growth pattern. It should be emphasised that where possible an entire node together with capsule should be atraumatically removed and processed by an experienced pathologist using fixatives chosen both to maintain nuclear and cytoplasmic detail and at the same time to be appropriate for phenotyping. It is to be anticipated that many of these features will be consolidated into World Health Organisation recommendations in the immediate future.\textsuperscript{14}

Accuracy was enhanced by adding immunostains using monoclonal antibodies. Here, consensus diagnosis improved between 2\% and 14\% in mucosal-associated lymphoid tumours, small lymphocytic or chronic lymphocytic leukaemia, those with lymphoplasmacytoid differentiation, high-grade or large B-cell tumours, nodal marginal zone and mantle-cell neoplasms. The striking advantage of these additional preparations in defining lineage was seen in the T-cell variants, whether precursor, anaplastic large or peripheral T-cell types. In these latter subtypes this information approximately doubled the number correctly identified over those using only sections stained with haematoxylin and eosin. Unfortunately, the need for these preparations cannot be predicted at the time of diagnosis, so that suitable planning must be made for the material to be studied using appropriate methods at a later date. It is noteworthy that flow cytometry is being increasingly employed, and that multidisciplinary groups managing these patients typically rely on this technology, using fresh tissue to avoid problems of fixation and processing.

One of the striking features of this study is that it confirms previous concerns that arose shortly after publication of the REAL Classification and were strongly reflected in the Cape Town experience, namely the importance of integrating all available information within a prognostic index, with particular emphasis given to patient data. This point is most remarkably demonstrated in primary mediastinal large B-cell tumours. However, the value of careful attention to clinical features, many of which reflect disease biology, cannot be underestimated. Logically it must, therefore, be acknowledged that previous preoccupation with purely microscopic features is
no longer acceptable. Correct stratification of patients to appropriate treatment programmes needs to integrate biochemical markers that include lactic dehydrogenase and additional imaging in order to document tumour distribution and bulk.

Assuming that optimum treatment protocols are used, the Cape Town experience again mimics that of the multicentre analysis in recognising four survival patterns. Consequently, overall 5-year survival exceeded 70% in patients with anaplastic large-cell lymphoma, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue, and the follicular lymphomas. A second group of these tumours occupied an intermediate position and comprised the nodal variant of marginal zone B-cell lymphomas, those with lymphoplasmacytoid differentiation and small lymphocytic lymphoma or its chronic leukemic equivalent. In the third category, where overall survival is approximately 50%, the majority of cases involved diffuse large B-cell neoplasms. In the remainder of cases this figure dropped to 30% and largely involved T-cell and mantle-cell lymphomas.

Although these results have improved over the last 5 years, they leave much to be desired. One area where new technology can be expected to improve outcome significantly is selection of treatment regimen based on inclusion of cellular and molecular biological data. In this context karyotyping or fluorescent in situ hybridisation that reveals the presence of chromosomal rearrangement or deletions is already proving valuable, both in diagnosis and in predicting results from conventional therapy. For example, the integration of such information into selection criteria for any particular treatment programme means that high-risk patients can rationally be assigned to intensive chemotherapy, often coupled with irradiation, and myeloprotection with haematopoietic stem-cell transplantation as first-line protocol management. A specific example will involve cases that at presentation have intermediate or high-risk features defined by the International Prognostic Index.7

A number of observations that emerge from the local study echo findings in other centres. The REAL Classification is therefore reproducible provided that the initial material is interpreted by an experienced pathologist, preferably one who is a regular participant in ongoing review workshops at international level. Secondly, cytomorphological and architectural features are no longer acceptable as the only criterion for diagnosis, with accuracy often being improved by inclusion of immunophenotyping and karyotypic analysis. Thirdly, there is growing acceptance that all relevant clinical information, including imaging and biochemical measurements, needs to be used in allocating patients to a risk category in the International Prognostic Index. Fourthly, and perhaps of particular relevance in developing countries, is the appreciation that work-up and management must be centred in multidisciplinary clinics that are at least nationally, and preferably internationally, accredited. This necessitates audit of every patient referred and peer-review of outcome in consecutively enrolled patients. Data accumulated in this way can be further monitored when it forms part of multicentre or collaborative studies.

In contrast, the previous practice of management by an occasional therapist, or even within a single-discipline practice, has shortcomings. These include over-treatment on the one hand, and failure to disclose the availability of innovative or investigational options on the other, which may lead to the equally unacceptable undertreatment. The clinical trials group of the South African Society of Medical Oncologists has taken a leadership role in this direction.8 Here ongoing local studies are being catalogued and efforts are being made to co-ordinate relevant approaches at national level with guidelines for conventional management, while concurrently advancing scientific standards through collaborative programmes. Issues that are suitable for such investigation include evaluation of topoisomerase inhibitors, high-dose chemoradiotherapy with myeloprotection using haematopoietic stem and progenitor cell transplants or anti-B-cell monoclonal antibodies. There are compelling advantages, particularly in the third world and as managed health care programmes permeate all levels of practice, for the small number of centres that have earned a reputation for excellence to be identified and designated as such. This step is needed to maintain standards, to disseminate information about what expectations exist with regard to treatment outcomes, and to provide a reliable base both for referral of new patients and to ensure that the guiding principles of interdisciplinary consultation are observed. Failure to act in this direction will fuel the downward spiral in understanding and therefore the correct contemporary management of patients with lymphoma.

CONCLUSION

In South Africa, as elsewhere in the world, the goal should be one of treating lymphoid malignancies with the intention of improving disease-free survival, or more directly, achieving the highest possible cure rate. The extent to which successful outcome is achieved depends on exacting diagnosis and stratification of patients to the most effective treatment option designed to capitalise on assignment-by-risk category, with appropriate weight being given to inclusion of clinical characteristics such as tumour progression and biology. The local experience, part of a much larger international study project, cogently argues for referring any patient with suspected lymphoma to an established multidisciplinary group without delay. In this way evaluation will be comprehensive and recommendations can be made about inclusion in peer-reviewed protocols.

Equally important is the obligation to monitor the response in each case objectively, so that appropriate changes to treatment can be made immediately there is any deviation from
anticipated outcome. Acceptance of these principles excludes the older practice of dabbling by occasional therapists, or even therapy given by single-discipline practices. These are largely curable neoplasms, and however unpalatable, there is no substitute for constant guidance — even supervision if appropriate — from the properly constituted and impartially functioning combined clinic.

I thank Fred Ulichnich and James Anderson, from the University of Omaha in Nebraska, for the separate statistical analyses of the cases from Cape Town; Patricia Marais and Gillian Ganz for help with the manuscript typing; and Christine Dölling for bibliographic assistance. Pauline Close, from the Department of Anatomical Pathology at the University of Cape Town, was the site pathologist. Carol Johnson, Radiation Oncologist, collated the clinical material. James O Armitage, Professor of Medicine, at the University of Nebraska Medical Centre, kindly reviewed the manuscript. These studies were supported by the Medical Research Council and the Cancer Association of South Africa.

References


Accepted 20 May 1999.
Editorial

Proceedings of the 12th Biennial Congress – South African Lymphoma Study Group

1. Background

More than 30 years ago these intriguing tumours arising in the lymphoreticular system started to attract attention. This was because of their relatively frequent occurrence and ready accessibility of involved tissue for biopsy and serial study to complement clinical and radiological course. There resulted effective treatments that improved quality of life and with constant refinement became a model to test combined modality regimens leading to evidence that potential cure was possible in many cases.

To generate an environment conducive for participation in these advances by means of worldwide scientific, academic and intellectual collaboration on one hand and providing optimum care for our patients on the other there was created the South African Lymphoma Study Group. Since inception Professor James O. Armitage provided encouragement for the imperative and constant stimulation by keynote presentations so that over more than two decades the initial small meeting, sponsored by Bristol–Myers, consolidated and grew steadily. Now registered as a national body these firmly entrenched meetings take place every second year bringing together a wide spectrum of local and overseas delegates sharing the common goal of improving understanding and outcome in these neoplasms of the immune system.

Even in an under-resourced country the ever-spreading recognition of how important precise diagnosis and accurate staging is in standardising risk-stratified protocols remains the central theme of these endeavours. Accordingly an interactive format has been retained that allows debate throughout the entire age spectrum, cuts across disciplines and, in this way, provides for constructive interchange between basic science, clinical haematology and immunology. There has always, simultaneously, been full accommodation of important contributions from the pharmaceutical industry with full integration of paramedical professions – most notably our nurses.

Particular effort has always been made to introduce and disseminate new knowledge exemplified by the rapidly emerging role of monoclonal antibodies and a range of small molecules that provide opportunities to test more precisely focused therapeutic interventions. Improving technology that enables distinctive signatures to be assigned to individual tumours using microchip gene array analysis provided impetus. Consequently classification becomes more biologically centred thereby supplementing traditional cytomorphology and histopathology and so providing a rationale for considering these neoplasms as reflecting the immune system in disarray. This burgeoning molecular genetic approach has become the focal point for entry into innovative therapies that extend to the use of radioconjugates and vaccination strategies.

Introduction was provided by Lucille Wood and her associates in an extension of data reported as part of the lymphoma reclassification project that brought together investigators from different parts of the world under the chairmanship of Professor James O. Armitage. Apart from geographical differences this experience documented good diagnostic concordance between trained haematopathologists when the revised European-American classification was used. Furthermore, unusual subtypes were
generally well accommodated within the system. Additionally, this participation provided a powerful incentive to register patients at a multidisciplinary or combined lymphoma clinic and an interim report describes outcome in Hodgkin and other lymphomas from this privately based academic centre in 253 consecutive referrals. Diagnosis was based on the World Health Organisation guidelines, prognosis assigned by the international index and therapy risk-stratified with results subject to appropriate statistical methodology. None of these patients underwent transplantation. It was concluded that, approached in this way, benefit accrued from the all-important centre effect with the proviso that matching figures are unlikely to apply, particularly in the Third World, outside such disciplined circumstances. This reflection is relevant since it is the reasonable expectation from patients and referring physicians alike that, since lymphomas are potentially curable, such comprehensive management will be regarded as standard of care. It follows logically that late referral and prior therapy will adversely affect performance status and compromise lifespan - these alternatives are inappropriate and need to be strongly discouraged.

The curability of follicular lymphoma was the keynote address by Jim Armitage who made the point that these entities had been widely regarded as incurable. However, if the cure is defined as remission that continues indefinitely and the patient eventually dies of another disease some will fall into this category. Furthermore, those with grade III act much like diffuse large B-cell lymphoma where disease eradication is frequent. This is in contrast to more indolent subtypes where there is variable response to initial therapy and here a significant proportion go on to benefit from haematopoietic stem cell transplantation.

Beacopp as standard for high-risk hodgkin lymphoma received authoritative comment from Volker Diehl in that consolidation radiation should be reserved to manage partial responses after an adequate number of anthracycline containing chemotherapy regimens or with residual nodal lesions. Dose and time intensification with an escalated version of this regimen further reduces the need for irradiation with the caveat that CT PET imaging may well discriminate between scar or viable tumour tissue. Importantly, initial benefits do not appear to be of set by later side-effects but the definitive answer awaits longer observation. These results should influence current approach including primary care specialists.

Telomeres and telomerase in leukaemia and lymphoma was reviewed by Glenda Davison who outlined the role of these structures in stabilising chromosomes and so, interactively, regulate cellular lifespan. Imbalance of the system is associated with the development of haematologic malignancy and improved understanding a potential basis for therapy targeting the telomere-telomerase complex.

The role of haematopoietic stem cell transplantation is discussed by Matthew Seftel in two contexts. In Hodgkin lymphoma patients failing to achieve sustained complete remission from chemotherapy become eligible for autografting. Not all such interventions are effective and new approaches include tandem procedures incorporating reduced intensity allogeneic grafts where the rationale is an immunologically mediated anti-lymphoma effect. Contrarily, chronic lymphocytic leukaemia continues to benefit from new therapeutic interventions despite which disease recurrence in relapse remains a major concern. Here, while autografting appears to have little benefit, allogeneic transplantation has curative potential that requires balance with procedure related complications that may be reduced with non-myeloablative conditioning.

Aids-related non-hodgkin and hodgkin lymphoma is noted by Gerhard Sissolak to have a relationship between incidence and progression of immunodeficiency. This poses a particular problem in sub-Saharan Africa with the rapidly escalating pandemic of human immunodeficiency viral infections where there is a counterbalancing effect from administration of highly active retroviral therapy making possible wider use of effective chemotherapy regimens. A number of unanswered questions include concomitant or sequential administration of antiviral and anti-lymphoma treatments, impact of resource allocation in Third World countries and the role of coinfection with oncogenic agents including human herpes and Epstein-Barr viruses. Consideration is given to epidemiology, pathogenesis as well as clinical features and treatment modalities in this situation.

Fine needle aspiration biopsy and flow cytometry in the diagnosis of lymphoma is considered by Guillaume Swart and his associates to have value in distinguishing reactive from neoplastic lymphoid populations when compared to the established practice of excision biopsy and histopathology but requiring ongoing evaluation. The argument is advanced that flow cytometry is rapid and objective in quantitatively and qualitatively documenting cell
surface characteristics. The utility of this approach has not been widely recognized and this is particularly true in South Africa. To better define the role of this combined procedure a series of patients were prospectively evaluated leading to the conclusion that, used proactively in a collaborative multidisciplinary setting, it may be possible to limit surgical procedures to specific situations requiring data that is not available from this specific intervention.

Castleman’s disease, hodgkin lymphoma and retroviral therapy is reported by Chris Kenyon and his associates to have an interesting interrelationship with the immunologic reconstitution that follows commencement of highly active retroviral therapy in patients with acquired immunodeficiency disease. Attention is drawn to the presentation after starting treatment perhaps reflecting an aberrant response to antigens that include the human herpes virus suggesting that proactive inclusion of drugs effective against these agents may be appropriate.

Novel proteosome inhibitors as a therapeutic strategy in lymphoma is reviewed by Akin Abayomi drawing on precedent from preclinical experiments and studies in myeloma for the presence of ubiquitin-proteosome pathway in modulating intracellular neoplastic processes that are protein dependent and can be modulated by inhibitors of the system. This concept is given focus by considering the introduction of this class of agents into the treatment of lymphoreticular malignancy.

Summary and conclusions, on behalf of the organising committee, was presented by Jim Armitage, Lucille Wood and Peter Jacobs. Their view was that this meeting re-emphasised the continued value of the South African Lymphoma Study Group, particularly on this continent, for all serious students of these tumours to share a common forum for presentation of their experience and contrast this with outcome in the first world. Such an opportunity remains valuable at a time when sub-Saharan Africa is reeling under the impact of an ever-accelerating academic of the acquired immunodeficiency disease with quaint approaches to treatment finding expression and prominent voices! Against this background it is reassuring to see that the reputation of a local scientific community survives as reflected in two ways. On the one hand is the sound quality of work being carried out in this country. On the other, endorsement is at a huge enough level to again bring some of the world’s unquestioned authorities to share in this important imperative. These deliberations are recorded in the accompanying publications.

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PROCEEDINGS OF THE 13TH BIENNIAL
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EDITORIAL

The South African Lymphoma Study Group came into being some 25 years ago to promote understanding of these tumours arising from the immune system at a time when they were also becoming increasingly recognised in this country. The impetus was found in a worldwide awareness of these entities and an early priority was to explore possible geographical differences. To foster such potentially relevant advances a scientific collaboration was established with Professor James O Armitage in Nebraska and that association has remained intact throughout subsequent years. Over time ever expanding interest, both locally and overseas, has led to the meeting being held, jointly, with other countries. These have included Israel, Belgium on two occasions, the United Kingdom and currently with Germany. A novel, and now distinctive, innovation has been to select a theme based on some particular characteristic having high profile at that time. This was exemplified, previously, by regarding these neoplasms as reflecting the immune system in disarray - a play on the recently developed technology of microchip gene array analysis. Another, and the current topic, is that of survivorship. Here, as treatment improved and survival lengthened, a surprising spectrum of underestimated and often overlooked side-effects have become recognised with the passage of time. These combine to have a varying and often adverse impact on quality of life. This phenomenon could be restated as an interrelationship between patient and the treating multidisciplinary team in seeking to maximise cure with optimal care. Appreciation of such enlightenment has altered focus to individualised management, rather than cases having purely statistical consideration. Of seminal importance is that this ethos accommodates personal choice on the basis of informed consent and balances often investigational regimens with ethical considerations.

One high profile intervention is that of immunohaematopoietic stem cell transplantation and the relative merits of autologous as opposed to allogeneic graft source still actively debated. Another, acute in the Third World, is resource distribution. Here, to help meet this need, the feasibility of duplicating traditional University Teaching Hospital facilities in the private sector was tested and found demonstrably realistic. Neither have the contributions of the pharmaceutical industry been neglected.
Accordingly the question before us this year was chosen to explore the extent to which new approaches, designed to circumvent anticipated side-effects, might be more effective and less toxic than past practices.

**Nomenclature** was the logical starting place where Jim Armitage set the tone by lucidly reviewing traditional approaches emphasising new diagnostic accuracy and subtyping within the broad scheme recently updated by the World Health Organisation giving focus to the peripheral T-cell variants. Integral to these established features is supplementation with additional immunophenotyping and, ever increasingly, both cytogentic and molecular methods. On such a basis signatures can be assigned not only to groups but individuals as the favoured approach for tailoring therapy. Guillaume Swart illustrated the role of fine needle aspiration biopsy as of value in separating benign from malignant lesions such as the breast or thyroid gland. In well trained hands the simple method can rapidly segregate these two distinctive pathophysiologic processes. However, when compared to the established practice of excision node biopsy, the additional information derived from histopathology, continues to undergo evaluation. The point was made that, particularly when tumours are atypical or polymorphic, confidence in distinguishing reactive populations is made more secure by use of simultaneous flow cytometry. With accumulating experience the need for surgical procedures can be reduced. This changing approach received emphasis from Ghulam Mufti discussing biological versus morphologic issues in the myelodysplastic syndromes – relevant since these entities may arise in the course of treated lymphoreticular malignancies. He made the point, elegantly, by stressing the increasing appreciation of contributions from unravelling mechanisms giving rise to genomic instability, cell cycle and immunotherapy using myeloid malignancies as the model.

**Pathogenesis** focused on the viruses where Diana Hardie showed how the presence of human gamma herpes viruses, causing persistent infection in the host, acquire genes from the recipient having the capacity to modify immune response as well as exerting oncogenic properties. Mervat Mattar outlined the causative association between hepatitis C virus and lymphoma in a proportion of non-Hodgkin variants drawing on her data from Egypt where high positivity rates in that population complicate management by necessary drug administration.
Glenda Davison noted that the immune system has the ability to control and destroy malignant cells well seen in the graft-versus-leukaemia effect evident when additional donor lymphocytes are infused following relapse from allogeneic transplantation.

Here dendritic cells are potent antigen presenters becoming activated after phagocytosis and processing foreign protein. During this process there is upregulation of MHC class II and other co-stimulatory antigens having the ability to recruit naive T cells. Recent evidence has shown that it is possible to load this population with tumour specific markers and so generate specific responses where that technology is promising in early clinical studies. Mahmoud Aljurf reviewed antigen and immune driven lymphoproliferative disorders with several of these possibly instigated by transformation of a polyclonal normal lymphocyte population to their monoclonal or neoplastic counterparts. The list of potential precipitating molecules continues to grow with the concurrent recognition that progression is a multistep process. During this transition, although clonal abnormalities may be recognised, some responsiveness to normal regulation of growth and differentiation persists so that the point of no return is hard to define. It is speculated that this may be the reason why similar morphologic or other subtypes occur concurrently in patients with profound immunosuppression.

Similarities are emerging in sub-Sahara where infection with the human immunodeficiency virus, leading to Acquired Immune Deficiency Syndrome, is rapidly gaining attention with its comparable profound defects in both innate and acquired arms often approaching that found in organ transplantation. This point was elaborated on in some detail by Gerhard Sissolak emphasising geographical differences based on a cohort of 512 consecutive individuals treated in a private academic centre. Very elegantly discussed by Hans Konrad Muller - Hermelink was the relationship of lymphoma in South Africa and, as he put it, the other world. This phenomenon was illuminated from his experience and large databases in treating and contrasting pathogenesis found in diffuse large cell lymphoma and Epstein-Barr viral related lymphoproliferations. Amplification of the need to establish worldwide cooperation in this area of research was given encouragement, particularly in the context of these meetings, by Volker Diehl who is a leading protagonist of information sharing.
desirable example as to just how much can be achieved with properly orchestrated national
efforts. His overview of advanced Hodgkin lymphoma in the context of ABVD and more recent
therapeutic options in fostering new international transatlantic studies was predictably insightful.
A point of some interest is that, in considering the management of these cases, principles emerge
that are pivotal to treat the underlying driving factor on the one hand and improve immune status
on the other. This may well be the mechanism incriminated by Alan Tooke in which a variety of
autoimmune disorders were shown to overlap with an increasing evolution to the malignant
counterpart of one or other lymphoma.

Clinical aspects are remarkably protean. Derek Miller reported that renal involvement is
probably more common than clinically suspected in most subtypes especially follicular and diffuse
large B-cell cases with autopsy studies showing upwards of 50% of cases with often-recognisable
features to be seen on CT scanning. Wide ranges of infiltrative patterns occur although most
typically in late stage disease and remain particularly useful in defining underlying pathology. An
important point is that reversibility is possible although some degree of chronic renal disease may
persist. Mark Sonderup explained how hepatic venous outflow obstruction gave rise to 3 distinct
categories with veno-occlusive syndrome at the level of the sinusoids and terminal - venules. In
contrast Budd - Chiari cases resulted from thrombosis in the hepatic veins with a timely reminder
that the former may complicate transplantation and the latter associated with thrombophilia.
Melvyn Letier described primary gastrointestinal lymphoproliferation most commonly found in the
stomach. Also highlighted were the mucosa-associated subtypes closely linked with Helicobacter
pylori with the observation that resolution may follow eradication of the organism. The small
intestine may often be silent for prolonged periods of time with recognition of these neoplasms
arising on the basis of overlooked gluten enteropathy. Central to many of these different organs
involved is demonstrating the extent of disease at diagnosis and, equally importantly, revealing
residual tumour as part of the staging and restaging program. The emerging role of positron
emission tomography combined with conventional CT scanning continues to be explored but there
was strong support from the academic faculty that functional as opposed to purely imaging needed
to be more widely accepted in this country. This point was convincingly presented by Vijay Dahya.
A hot topic was the management of cytopenia in those who are retroviral positive even before the emergence of the Acquired Immune Deficiency Syndrome and this is a rapidly escalating challenge as this viral epidemic relentlessly escalates.

Matthew Seftel stressed the importance of fever in neutropenic cancer patients as representing a medical emergency having a significant incidence of overwhelming infection and death. The spectrum of microbiological pathogens varies both temporarily and geographically so that universal recommendations for antimicrobial therapy are still challenging. Nonetheless unifying principles are clear with areas for resolution being the place of prophylactic antibiotics, safe treatment in an outpatient setting and empiric regimens. This section was completed by Christina Stephane defining the paediatric perspective where these tumours comprise 10% of childhood cancers and up to 17% in teenagers. Hodgkin lymphoma is seen in much younger ages in the developing countries with a male predominance and histologic subtypes varying between ethnic groups. A remarkable observation was that under 15 years the presence of Epstein-Barr viral markers were suggestively associated with favourable survival although interracial variation recognised. Disturbingly the non-Hodgkin variants increase with age and shift to a more aggressive variants. Particular effort is needed to identify effective low toxicity protocols, avoid radiotherapy and retain compliance. Across all of these important variables is the escalating frequency of late effects including infertility, impaired cognitive performance and emergence of secondary malignancies.

_Treatment_ is increasingly multimodality. This shift in concept and practice continues to move treatment away from the old and largely inflexible concept of Third World practices as regarding one regimen as appropriate for all! In its place is the use of prognostic factors to individualise regimens that will typically link agents having different mechanisms of action. Choices range from traditional cytotoxic drugs through rapid acceleration in the use of monoclonal antibodies to a changing perspective for bone marrow transplantation in one or other of its various forms. In the most traditional sense Vernon Louw elegantly summarised the current status for Waldenstroms macroglobulinaemia. Asim Belgaumi instructively used his database in very young children with Hodgkin lymphoma as a model to argue for causal infectious association.
consequences making the telling point that, while information is relatively restricted, differences are quite clear between developed countries and under resourced areas of the world.

Marcel Reiser presented pharmacodynamic studies on how rituximab could be escalated in the treatment of high-grade non-Hodgkin lymphomas. Constructively there was balance between the effects of increasing density, with or without intensity, of the conventional drug regimen against higher levels of monoclonal antibody with utility in elderly having poor prognosis. Along similar lines Andres Engert concisely used the powerful German Hodgkin Lymphoma Study Group database to update attendees on the relative merits of established chemotherapy programmes such as ABVD and more aggressive combinations exemplified by BEACOPP in both standard and escalated forms. Notable was the tour de force for using phenotype to introduce not only rituximab as a relatively new development in exploring toxin mediated selective immunotherapy. In keeping with the theme of the meeting to reflect on late effects of treatment and survivorship in this distinctive lymphoma.

Another monoclonal antibody of proven value in these tumours of the lymphoid system was the way Anders Österborg demonstrated the emerging role of alemtuzumab in targeted therapy. Along these lines immunotherapy appears to have considerable potential and the principles lucidly described by Said Dermime in both a mouse model showing effectiveness of the vaccine as well as leukaemia.

Not surprisingly bone marrow transplantation remains an important option with David Brittain discussing its role in relapsed Hodgkin lymphoma. Perspective - as counterpoint - emerged from the eastern Mediterranean and North Africa in the presentation by Mahmoud Aljurf. Jackie Thompson related her experience of myeloablative allografting for the low-grade lymphoid malignancies and thought-provoking presentation by Devan Moodley reported early but clear benefit in patients with multiple sclerosis having progressive disease.

Penultimately Mike Webber provided a timely reminder of how psychoneuroimmunological influences impact outcome in quality of life. The point was how ethics and changes in neuropsychiatric functions can be seen as a thread running through these practices often providing an anchor for accommodating to these different periods - important in the concept of survivorship.
Impressive was the discussion by Marc Blockman of how significant adverse drug events were an ever-present risk in almost all modern treatment programs. These important considerations were given strong emphasis by Tony Behrman who brought finality to our deliberations underlying the importance of ethics, ethical rules and illustrated these in the context of the modern day haematologist.

Summary and closure by Andres Engert emphasised the pride of place enjoyed by these intercontinental biennial meetings. They continue to bring together all serious students of the constantly expanding group of tumours arising from the immune system and do so at a time when sub-Saharan Africa is reeling under the impact of an ever-accelerating epidemic of the acquired immunodeficiency syndrome. The latter is, sadly, still a beneficiary of most unusual therapeutic approaches to treatment finding expression in prominent voices! Against this background it is reassuring to see that the reputation of the local scientific community survives as reflected in two ways. On the one hand the sound quality of work presented from the South African workers. On the other the endorsement that has again brought many eminent authorities from all over the world to participate in what is, unquestioned by any standards, a stellar faculty. This increasingly popular and highly effective forum achieved a degree of interaction that was rewarding for delegates and faculty alike. It is this ethos that has defined the recognised benefits of continuing with the alternate year symposia.

Despite tumultuous changes that range from the American presidency through worldwide financial collapse, to disasters in almost every country there is nevertheless an obligation felt by the organising committee to maintain this forum for the benefit of patients and the professional multidisciplinary teams alike. Accordingly in October of 2010 the South African Lymphoma Study Group will reconvene being hosted jointly with our colleagues from the Netherlands and participants from Belgium, Saudi Arabia and other parts of the world in what will be the 14th biennial Congress.

The initial announcement and further information is available by contacting Reyhanah Trevor:

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Abstract #353

Chimeric Anti-CD20 Monoclonal Antibody (Rituximab;Mabthera) in Remission Induction and Maintenance Treatment of Relapsed or Resistant Follicular Non-B-Cell Lymphoma: Final Analysis of a Phase III Randomized Intergroup Clinical Trial

Diwakar V. Van Ginkel,1 Ivana Toledovovic,1 Cynthia Rozewicz,1 Robert K. Marcus,3 Max Wolf,4 Eva Kimby,1 Anton Hagenbeck,12* EORTC Lymphoma Group, Academic Medical Center, Amsterdam, Netherlands; 2Hovon; 3EORTC Data Center; 4German-Italian follicular Lymphoma and Leukemia Group; 5North American Lymphoma Group.

Background. Last year we reported interim results of a randomised phase III trial in patients with relapsed refractory stage IV M-II positive follicular NHL demonstrating that the addition of rituximab (R) to CHOP chemotherapy for remission induction as well as the addition of rituximab induction prior to CHOP chemotherapy significantly improved the clinical outcome (Blood 2004:104:169a). Based on these interim results, randomisation to the induction part of the trial was stopped and the protocol was amended. Study design. Patients with stages III or IV follicular lymphoma at initial diagnosis and relapsed after or resistant to a maximum of two non-chemotherapeutic (ie, systemetic chemotherapeutic) regimens, were randomized to remission induction with either 6 cycles of CHOP (375 mg/m2 once every 3 weeks) or CHOP + R (375 mg/m2 as a bolus at day 1 of each cycle of CHOP). Those with a complete or partial remission after 6 cycles of therapy underwent a second randomization to no further treatment (obsevational) or maintenance treatment with R (375 mg/m2 once every 3 months) until relapse or for a maximum period of 2 years. At the time of the preplanned second interim analysis (February 2004), 461 patients had been included. Of these, 369 could be evaluated for response (185 CHOP; 181 R-CHOP). Both treatment arms yielded similar initial response rates (CHOP: 55%; R-CHOP: 52.5%), but highly significant differences in CR rates between CHOP (18.1%; R-CHOP: 30.4%; p<0.0004). Of 319 patients randomized for maintenance treatment, 268 were evaluable (132 observation; 136 R maintenance). A highly significant improvement was observed in progression-free survival in R maintenance, when compared with observation arm (median PFS 38 vs 15 months; p=0.0001). At that time there was no impact on OS: the observed difference (in favor of R maintenance) was not significant for any interim analysis. Toxicity of CHOP and R-CHOP induction and maintenance (CR rates as compared to CHOP) was manageable with minimal toxicity. These results of the planned second interim analysis showed that the formal criteria for stopping the trial had been met. It was concluded that this is the first trial to show that: 1) in patients with relapsed refractory follicular lymphoma R-CHOP induction regimens in a high-risk patient population increase in CR rate as compared to CHOP; 2) rituximab maintenance treatment significantly improved PFS in patients responding to induction treatment. The final analysis of the study results will be performed in September 2005, demonstrating that a follow-up of at least 18 months. The final results will be presented at the meeting.

Abstract #354

Low Dose Radiation Induces a Highly Effective p53 Response and Rapid Tumor Regression in Pancreatic Lymphoma. Laurent Kroopp,1 Rick L. Haas,2 Samne de Kemp,3 Annegien Broekx,4 Laura J. van 't Veer,5 Desphene de Jong,6* (from, by Marie Jose Kereste) Pathology, The Netherlands Cancer Institute, Amsterdam, Netherlands; 2Radiotherapy, The Netherlands Cancer Institute, Amsterdam, Netherlands; 3Experimental Therapy, The Netherlands Cancer Institute, Amsterdam, Netherlands.

Results. We administered a fractionated field radiation therapy with 30 to 40 Gy in a usable local treatment for follicular lymphoma (FL) that is routinely used in clinical practice. We previously showed that low dose radiation therapy (2Gy Gx, days 1 and 4) induced a radiosensitive population in up to 10% of patients (Bouma et al, IJC, 2003). However, the biological mechanism of this extremely effective response is not known. To study the molecular response to low dose radiation therapy in FL, gene expression profiling using 32K spotted oligo arrays from lymphoblastoid cDNA samples taken before treatment and 24 hours after the second dose of 2Gy irradiation, in 15 patients. The clinical response was excellent (10 CR, 5 PR). In all patients, a major and consistent induction of p53 and p53 target genes was seen, accompanied by repression of 14 out of 9000 genes (3%). p53 upregulation, p53-mediated proliferation arrest and apoptosis was substantiated using immunohistochemistry with dramatic increase of p53 protein levels in B-cells, T-cells and accessory cells. There was also a significant increase in the numbers of cleaved caspase 8 positive cells (death receptor pathway apoptosis) with a minor increase of cleaved-caspase 3 positive cells and morphological features of apoptosis, suggesting a relatively early stage in the apoptosis pathway.

The primary induced genes were an "immune signature", with a whole set of biologically meaningful genes related to macrophages (e.g., CD68, TH14), ThI immune response (e.g., IL18, ICOS2, 10, 11), clearance of apoptosis cells (e.g., CYP4A), MHC-I expression (HLA-A, B, CDD) and death receptor pathway. The immunohistochemical analysis did not show an increase in T-cell subsets and macrophages density, rather suggesting an activation or differentiation of resident macrophages by radiation and/or apoptotic cells that contributed to the death and clearance of tumour cells. These insights may have important implications for modulation of the cancer-related immune response and for immunotherapeutic approaches in FL.

References

[1817] Outcome of Patients Developing GVHD after DLI for CML Relapse from HLA-Identical Sibling or VUD HSCT. Session Type: Poster Session 21-II

Yves Chalandon, Christoph Schmid, Kimmo Porkka, Alvaro Urbano-Ispizua, Bernd Hertenstein, Francesco Dazzi, Eduardo Olavarria, Jane Appleby, Per T. Ljungan, Antonius V.M.B. Schattenberg, Stig Lenhoff, Peter Jacobs, Jurgen Finko, Dietgar W. Niederwieser, Cesare Guglielmi On Behalf of the Chronic Leukemia Working Party (CLWP) of the European Group for Blood and Marrow Transplantation (EBMT)

Using data submitted to the EBMT registry, we analyzed outcome on 344 patients (pts) who had received donor lymphocyte infusions (DLI) for relapse after allogeneic hematopoietic stem cell transplantation (HSCT) for chronic myeloid leukemia (CML) in 31 centers. 113/344 pts (33%) developed acute graft-versus-host disease (aGVHD) a median of 50 days post DLI (max grade: 1=42, II=30, III=31, IV=5)(60% grade II-IV). Organs involved (%): skin (88), liver (42), gut (30). Median age was 38 (4-59). 56% of pts were male, 62 transplants were HLA-identical sibling and 51 unrelated. 74 were T-cell depleted, 92 transplanted in CP1, 21 beyond CP1. Relapse was molecular in 19 pts, cytogenetic in 31, hematomatological in 49, accelerated or blastic in 12. Median initial cell dose was $10^7$CD34 cells/kg (0.01-32), median number of DLI was 1 (1-10). aGVHD was treated with prednisone in 92% of pts, CSA in 52%, ATG and monoclonal antibodies in 2% and other in 19%. aGVHD resolved in 53% of the pts within a median of 83 d (7-846). 82/344 pts (24%) had chronic GVHD (cGVHD)(30 limited, 50 extensive, 2 not specified), of those 46 (56%) following aGVHD post DLI. Organs involved (%): skin (75), liver (35), lungs (13), mouth (43), eyes (22) and gut (9). Median age was 35 (6-58). 51% were male, stem cell source was PB in 15% and marrow in 85%, 43 underwent HLA-identical sibling HSCT and 36 unrelated donor HSCT. Forty-three were T-cell depleted, 66 transplanted in CP1, 16 beyond CP1. Relapse was molecular in 21 pts, cytogenetic in 29, hematological in 22, accelerated or blastic in 7. Median initial dose was $10^7$CD34 cells/kg (0.05-40), median number of DLI was 1 (1-7). 61 pts are alive with a median follow-up of 50 mth. Treatment was with steroids in 83% of pts, CSA in 58%, MMF in 20%, thalidomide in 15%, photopheresis in 15%, PUVA in 10% and other in 17%. cGVHD resolved in 39% of the pts within a median of 354 d (44-1588). The estimated 5-y OS post-DLI was significantly lower in pts who developed aGVHD post-DLI, 61 ± 10% vs 74 ± 7% in the one that did not, p=0.007 and also a tendency to have a lower 5-y EFS, 58 ± 10% vs 65 ± 7%, p=0.19. Median duration of response to DLI in aGVHD pts was 4 y. aGVHD post-DLI did not influence the relapse rate (5 ± 5% vs 6 ± 5% in the absence of aGVHD). 5-y DLI related mortality was significantly higher in aGVHD pts, 31 ± 8% vs 4 ± 4%, p=0.000071. On the other hand, pts that developed cGVHD post-DLI had a tendency to have a better 5-y OS and EFS, 74 ± 11% and 71 ± 11% respectively vs 69 ± 5% and 62 ± 7% in those that did not, p=0.32 and 0.09. This was related to a tendency to lower incidence of relapse, 2 ± 3% in pts with cGVHD vs 9 ± 6% without, p=0.2. DLI related mortality was not different, 11 ± 8% vs 10 ± 5%, p=0.77. aGVHD post-DLI for CML relapse is mainly of advanced stage and negatively influence OS and EFS with a higher DLI related mortality cGVHD post-DLI is mainly extensive, but pts with cGVHD tend to have better outcome with better 5-y OS, EFS and less relapse than those without, although this was not statistically significant.

Abstract #1817 appears in Blood, Volume 106, Issue 11, November 16, 2005 Keywords: Relapse|Acute graft-versus-host disease|Chronic graft-versus-host

Sunday, December 11, 2005 9:15 AM
Poster Session: Acute and Chronic GVHD (9:15 AM-7:30 PM)
Marrow versus peripheral blood for geno-identical allogeneic stem cell transplantation in acute myelocytic leukemia: influence of dose and stem cell source shows better outcome with rich marrow

Norbert C. Gorin, Myriam Labopin, Vandersand Focha, William Arcese, Moriel Beksa, Eliane Gluckman, Olle Ringden, Tapani Ruutu, Josy Reilffers, Giuseppe Bandini, Michele Falda, Panagiotis Zikos, Roell Willemen, and Francesco Frassoni, for the Acute Leukemia Working Party (ALWP) of the European Cooperative Group for Blood and Marrow Transplantation (EBMT)

Several studies have compared bone marrow (BM) and peripheral blood (PB) as stem cell sources in patients receiving allografts, but the cell doses infused have not been considered, especially for BM. Using the ALWP/EBMT registry, we retrospectively studied 861 adult patients with acute myelocytic leukemia (AML), who received a non-T-depleted allogeneic BM (n = 515) or mobilized PB (n = 366) standard transplant, in first remission (CR1), from an HLA-identical sibling, over a 5-year period from January 1994. The BM cell dose ranged from 0.17 to 29.6 x 10^6/kg with a median of 2.7 x 10^6/kg. The PB cell dose ranged from 0.02 to 77 x 10^6/kg with a median of 9.3 x 10^6/kg. The median dose for patients receiving BM (2.7 x 10^6/kg) gave the greatest discrimination. In multivariate analyses, high-dose BM compared to PB was associated with lower transplant-related mortality (RR = 0.51; 95% CI, 0.39-0.98; P = .04), better leukemic-free survival (RR = 0.65; 95% CI, 0.46-0.91; P = .013), and better overall survival (RR = 0.64; 95% CI, 0.44-0.92; P = .016). The present study in patients with AML receiving allografts in first remission indicates a better outcome with BM as compared to PB, when the dose of BM infused is rich. (Blood. 2003;102:3043-3051)

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Introduction

In the past decade, the use of peripheral blood (PB) as a source of hematopoietic stem cells for allogeneic transplantation has increased considerably. A large number of retrospective studies,4-6 several prospective and randomized,7-10 have compared the outcome of patients receiving allografts with bone marrow (BM) versus PB, using a family identical sibling; in all studies PB has resulted in faster engraftment and shorter hospital stay. In most studies, with the notable exception of the EBMT study,8 the incidence and severity of acute graft-versus-host disease (aGVHD) has been similar with PB and BM; PB on the other hand has been associated with more chronic GVHD (cGVHD). Finally, the outcome with PB, in terms of leukemia-free survival (LFS) and overall survival (OS), has been identical to BM and sometimes superior.14 All things considered, for practicality reasons, PB has therefore become a first choice for many transplantation teams. It has been postulated that the benefit brought by PB was at least in part due to the higher cell dose infused to the recipient when compared to BM.15

In the same period, other studies on BM and PB transplantations have drawn attention to the importance of the dose of stem cells infused; a lower transplant-related mortality (TRM) and also in some studies a lower relapse rate after transplantation have been observed in patients undergoing allografting13,14 or autografting15-19 with higher BM cell doses.

In the present retrospective study based on the ALWP/EBMT registry, we compared the outcome of patients with acute myelocytic leukemia (AML) receiving allografts in first remission (CR1) using BM or PB as a source of stem cells, focusing on the dose of stem cells infused. We found a significantly better outcome in patients receiving higher BM cell doses (rich marrow), when compared to the others, that is, patients receiving low-dose BM or high- or low-dose PB.

Patients and methods

Data collection and patient selection

The European Blood and Marrow Transplant (EBMT) Registry is a voluntary working group of more than 500 transplantation centers. Participants are required once a year to report all consecutive transplantations and follow-up. The Acute Leukemia Working Party (ALWP) of the EBMT is in charge of validating and checking submitted data to ensure data quality.

From the Centre international greffes de moelle, Hôpital Saint-Antoine, AP-HP, European Data Management Office of the EBMT, UPRES EA 1838, and Centre de recherche Claude Bernard sur la thérapie cellulaire, Université Paris VI, Paris, France; Hôpital St Louis, Paris, France; Universita La Sapienza, Rome, Italy; ibni Sina Hospital, Ankara, Turkey; Huddinge University Hospital, Stockholm, Sweden; Helsinki University Central Hospital, Finland; Hôpital Haut-Lévêque, Pessac, France; Hospital San Orsola, Bologna, Italy; Azienda Ospedaliero S. Giovanni, Torino, Italy; Patras University Medical School, Patras, Greece; Leiden University Hospital, the Netherlands; and Ospedale San Martino, Genova, Italy.


Supported in part by EBMT funds and Association Claude Bernard.

A complete list of EBMT members contributing to the ALWP appears in the Appendix.

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This study included 881 patients with AML, older than 16 years of age, who received a non-T-depleted allogeneic BM (n = 515) or mobilized PB (n = 366) standard transplant, in CR1, from an HLA-identical sibling, over a 5-year period from January 1994 (date of the first allogeneic PB transplantation) to January 2001. The dose of cells infused with the graft was in nucleated cells per kilogram. The study was approved by the EBMT review board.

**End points of the study**

*Hematopoietic recovery.* Neutrophil and platelet recoveries were analyzed separately and defined by a neutrophil count equal to or more than 0.5 X 10^9/L for 3 consecutive days and a platelet count equal to or more than 50 X 10^9/L for 7 consecutive days with no platelet support, respectively. The median time to recovery was calculated using the product limit method.

*Mortality and relapse.* Transplant-related mortality (TRM) was defined as nonleukemic deaths. Relapse incidence (RI) was defined on the basis of morphologic evidence of leukemia in BM or other extramedullary sites. To evaluate the probability of relapse, patients dying either from direct toxicity of the procedure or from any other cause not related to leukemia were censored. Leukemia-free survival (LFS) was defined as the time interval from transplantation to the first event (either relapse or death in complete remission).

GVHD. Acute GVHD (aGVHD) was diagnosed and graded at each transplantation center according to Seattle criteria. Only patients with grade II or superior were considered as having aGVHD. For chronic GVHD (cGVHD), only patients surviving without relapse for more than 100 days after transplantation with sustained donor engraftment were considered as evaluable; cGVHD was defined according to standard criteria (limited and extensive).

**Statistical analyses**

All analyses were performed with the SPSS statistical analysis program (SPSS, Chicago, IL). Values reported for quantitative variables were median and range. The following patient or graft characteristics were analyzed for their potential prognostic value on each of the outcomes: patient's and donor's characteristics (age, sex, and sex matching), disease factors (white blood cell count at diagnosis, French-American-British classification, interval from diagnosis to CR1, interval from CR1 to transplantation), and transplant-related factors (source of stem cells, nucleated cell dose infused per kilogram, year of transplantation, nature of the conditioning regimen including or not total body irradiation). For these prognostic analyses, continuous variables were categorized according to the median value. To compare the distribution between the subgroups of patients, we used the χ² test for categorical variables and the nonparametric Mann-Whitney U test for continuous variables.

Patients were censored at the time of relapse or the last follow-up. Probability of DFS, RI, TRM, and OS were estimated by the product-limit method. The significance of differences between curves was estimated by the log-rank test (Mantel-Cox). All variables associated with outcome with a P value of less than .1 in univariate analyses and characteristics statistically different (P < .05) between subgroups of patients were included in a multivariate analysis. Because a center effect had been observed in a previous EBMT study in patients receiving a BM transplant for AML in CR1, all further multivariate analyses were adjusted on center.

| Table 1. Distribution of patients by source and dose of nucleated cells/kg infused |
|-------------------------------|---------------------------------|-------------------|-------------------|
|                               | BM                             | NC more than 2.7 X | NC more than 2.7 X |
|                               | 10^9/kg, n = 258              | 10^9/kg, n = 257   | 258              |
| Age, y (range)                |                                 |                   |                   |
| Patients                      | 35 (10-60)                    | 35 (10-60)        | 35 (10-60)       |
| Donors                        | 35 (10-60)                    | 35 (10-60)        | 35 (10-60)       |
| Patient sex (%)               |                                 |                   |                   |
| Male                          | 128 (51)                      | 130 (51)          | 130 (51)         |
| Female                        | 124 (49)                      | 124 (49)          | 124 (49)         |
| Donor sex (%)                 |                                 |                   |                   |
| Male                          | 128 (51)                      | 130 (51)          | 130 (51)         |
| Female                        | 124 (49)                      | 124 (49)          | 124 (49)         |
| White blood cell count at diagnosis, X 10^9/L | 11 (0.2-420) | 10.6 (0.7-500) | 13.5 (0.7-710) |
| FAI classification (%)        | 3 (1)                         | 1 (0.4)           | 3 (1)            |
| M0                            | 46 (20)                       | 53 (23)           | 52 (16)          |
| M1                            | 67 (28)                       | 84 (39)           | 111 (54)         |
| M2                            | 13 (5)                        | 9 (4)             | 11 (3)           |
| M3                            | 58 (25)                       | 46 (23)           | 81 (25)          |
| M4                            | 37 (16)                       | 29 (13)           | 52 (16)          |
| M5                            | 6 (2)                         | 10 (4)            | 13 (4)           |
| M7                            | 4 (2)                         | 1 (0.4)           | 4 (1)            |
| Cytogenetics, n (%)           | n = 125                       | n = 115           | n = 126          |
| Good                          | 21 (17)                       | 15 (13)           | 21 (17)          |
| Intermediate                  | 95 (76)                       | 93 (91)           | 96 (79)          |
| Poor                          | 9 (7)                         | 7 (6)             | 9 (7)            |
| Interval diagnosis-CR1, d (range) | 40 (12-305) | 43 (17-400) | 41 (17-422) |
| Interval CR1-transplantation, d (range) | 99 (13-365) | 94 (14-875) | 92 (12-594) |
| TBI, %                        | 45                             | 57                | 57               |
| NCs infused, X 10^9/kg (range) | 2 (0.17-2.7) | 3.68 (2.7-23) | 9.3 (2.2-77) |
| aGVHD grades II-IV, %         | 38                             | 35                | 36               |
| aGVHD grades III-IV, %        | 13                             | 9                 | 12               |
| Follow-up, mo (range)         | 33 (1-78)                     | 35 (1-81)         | 16 (1-72)        |

NC indicates nucleated cells; —, not applicable.
Results

Patient populations in relation to hematopoietic stem cell doses infused and univariate analyses

The outcome was identical when comparing BM and PB transplantations: At 2 years, the TRM with BM and PB was 22% ± 2% and 22% ± 2% (P = .98), the RI 19% ± 2% and 22% ± 3% (P = .61), the LFS 63% ± 2% and 61% ± 3% (P = .72), and the OS 66% ± 2% and 66 ± 3% (P = .82), respectively.

The BM cell dose ranged from 0.17 to 29 × 10^9/kg recipient weight with a median of 2.7 × 10^9/kg. The PB cell dose ranged from 2.2 to 20 × 10^9/kg with a median of 9.3 × 10^9/kg.

There was no difference for outcome when taking the median dose of PB cells infused as a cutoff within the PB group: The 3-year LFS was 64% ± 6% in patients receiving doses below the median and 59% ± 7% for those receiving doses above the median (P = .76). For patients receiving BM, the median dose (2.7 × 10^9/kg) gave the greatest discrimination for all end points studied.

We therefore studied 3 populations of patients: those receiving higher doses (above median) of marrow (n = 257), those receiving lower (below median) doses of marrow (n = 258), and those receiving PB whatever the dose (n = 366).

Table 1 gives the distribution of the 3 groups for disease and transplantation characteristics; the only differences concerned the year of transplantation, which was more recent for PB, and more TBI and a trend for fewer female donor-to-male recipient combinations in the group receiving higher doses of BM.

The distribution was even for patient age and sex, white blood cell count at diagnosis, FAB classification, and pretransplantation intervals.

Information on cytogenetics was available for 366 patients. In keeping with our previous work, 24-27 patients were in the good-risk category (t(15;17), t(8;21) and inv(16), 25 in the poor-risk category (abnormality 5 or 7, 11q-), and 284 in the intermediate-risk category. The number of missing values was too high to include cytogenetics in the multivariate analysis. However, the distribution by cytogenetics over the 3 groups of patients was even.

Recovery of polymorphonuclear cells (PMNs) to 500/mm^3 with high-dose BM, low-dose BM, and PB occurred at days 18 (range, days 10-39), 19 (range, days 10-53), and 14 (range, days 10-42), respectively. The differences were statistically significant between rich and poor BM (P = .009) and PB and poor BM or rich BM (P < 10^-4 for both). Recovery of platelets to 50 000/mm^3 with high-dose BM, low-dose BM, and PB occurred at days 24 (range, days 12-385), 27 (range, days 12-235), and 17 (range, days 7-343), respectively. The differences were statistically significant between rich and poor BM (P = .02), PB and poor BM (P = .0002) or rich BM (P < 10^-4).

Table 2. Outcome of patients by source and dose of nucleated cells/kg infused

<table>
<thead>
<tr>
<th>BM, %*</th>
<th>NC less than 2.7 × 10^9/kg</th>
<th>NC more than 2.7 × 10^9/kg</th>
<th>PB, %*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-y outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LFS</td>
<td>54 ± 3</td>
<td>72 ± 3</td>
<td>61 ± 3</td>
<td>.17</td>
</tr>
<tr>
<td>RI</td>
<td>25 ± 3</td>
<td>14 ± 2</td>
<td>22 ± 3</td>
<td>.23</td>
</tr>
<tr>
<td>TRM</td>
<td>27 ± 3</td>
<td>17 ± 2</td>
<td>22 ± 2</td>
<td>.23</td>
</tr>
<tr>
<td>OS</td>
<td>59 ± 3</td>
<td>74 ± 3</td>
<td>66 ± 3</td>
<td>.88</td>
</tr>
<tr>
<td>1-y cGVHD</td>
<td>43 ± 5</td>
<td>40 ± 5</td>
<td>50 ± 4</td>
<td>.002</td>
</tr>
</tbody>
</table>

*Values given as mean ± SD.
Figure 2. TRM of patients receiving transplants with high-dose BM, low-dose BM, or PB.

None of the other studied factors (patient and donor age and sex, female donor to male recipient, year of transplantation, and use of TBI) influenced any component of the outcome.

Discussion

In the past 5 years several prospective randomized studies have compared BM and PB as alternative sources of stem cells for allogeneic stem cell transplantation using HLA-identical siblings. Conclusions from these studies have been that PB is associated with faster engraftment, similar or greater incidence of aGVHD, and higher incidence of cGVHD. LFS and OS have been found identical or even better with PB. All these studies have combined transplantations for several hematologic malignancies. A more limited number of retrospective studies have suggested poorer results with PB in more risky situations, such as with mismatched related donors, unrelated donors, or more advanced diseases. Also, it has been recently suggested that results may vary for different diseases, with the observation in transplantations using unrelated donors of similar outcome with BM and PB in AML contrasting with a worse outcome with PB in acute lymphocytic leukemia (ALL). The minimum doses of cells to ensure safe engraftment have been established very early with the development of BM and then PB transplantation. For allogeneic stem cell transplantation, it is generally accepted that a BM graft should contain more than \(2 \times 10^7\) nucleated cells/kg and a PB graft (as initially defined in the setting of autologous PB stem cell transplantation) more than 2 or even better \(5 \times 10^7\) CD34+ cells/kg. These thresholds have been established from observations on the kinetics of engraftment and, with PB, infusion of doses more than \(5 \times 10^7\) CD34+ cells/kg has been shown not to further reduce the duration of aplasia. Only recently, however, has attention focused on the possibility that increasing the doses of stem cells above these thresholds might not only reduce the TRM but also and more unexpectedly reduce the relapse/progression rate of the underlying disease. In the field of AML specifically, in the context of autologous BM transplantation with BM purged by mafosfamide, the dose of stem cells infused has been identified as an important prognostic factor for outcome.

Table 3. Prognostic factors other than cell dose influencing outcome, \(P\) (log-rank test)

<table>
<thead>
<tr>
<th>Factor</th>
<th>LFS</th>
<th>RII</th>
<th>TRM</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient sex</td>
<td>.91</td>
<td>.69</td>
<td>.78</td>
<td>.53</td>
</tr>
<tr>
<td>Donor sex</td>
<td>.86</td>
<td>.1</td>
<td>.09</td>
<td>.88</td>
</tr>
<tr>
<td>Female to male</td>
<td>.54</td>
<td>.1</td>
<td>.001*</td>
<td>.16</td>
</tr>
<tr>
<td>FAB M5 versus other</td>
<td>.88</td>
<td>.83</td>
<td>.86</td>
<td>.7</td>
</tr>
<tr>
<td>TBI</td>
<td>.72</td>
<td>.79</td>
<td>.48</td>
<td>.57</td>
</tr>
<tr>
<td>Patient age</td>
<td>.05</td>
<td>.56</td>
<td>.001*</td>
<td>.02</td>
</tr>
<tr>
<td>Donor age</td>
<td>.0001*</td>
<td>.28</td>
<td>.01</td>
<td>.01</td>
</tr>
<tr>
<td>Year of transplantation (before 1997 versus after 1997)</td>
<td>.26</td>
<td>.16</td>
<td>.79</td>
<td>.52</td>
</tr>
<tr>
<td>Interval diagnosis-CR1, d</td>
<td>.68</td>
<td>.38</td>
<td>.003*</td>
<td>.02</td>
</tr>
<tr>
<td>Interval CR1-transplantation, d</td>
<td>.16</td>
<td>.52</td>
<td>.001*</td>
<td>.14</td>
</tr>
<tr>
<td>White blood cell count at diagnosis</td>
<td>.34</td>
<td>.82</td>
<td>.77</td>
<td>.51</td>
</tr>
<tr>
<td>Center</td>
<td>.001*</td>
<td>.22</td>
<td>.03</td>
<td>.01</td>
</tr>
</tbody>
</table>

*One class grouping all centers performing fewer than 10 transplantations during the period.
†Significant difference.

Table 4. Causes of death

<table>
<thead>
<tr>
<th>Cause</th>
<th>BM less than (2.7 \times 10^9) kg, %</th>
<th>BM more than (2.7 \times 10^9) kg, %</th>
<th>PB, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Failure/rejection</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Infection</td>
<td>21</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Intestinal pneumonitis</td>
<td>2</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>GVHD</td>
<td>28</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td>Leukemia</td>
<td>43</td>
<td>45</td>
<td>40</td>
</tr>
<tr>
<td>Other</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
for various hematologic malignancies, a greater dose of nucleated or progenitor CD34+ cells has been shown to reduce fungal infections and TRM and to result in a better OS. As an example in 212 patients who received a transplant of an unmanipulated graft from an HLA-identical sibling donor, 10 year survival and 180-day TRM were, respectively, 64% and 19% for patients receiving a CD34+ cell dose of 3 x 10^6/kg or more and 40% and 37% for the other. EBMT similarly has recently reported on the impact of the dose of BM on the outcome of patients receiving allografts for AML with a geno-identical family donor; higher doses of cells infused expressed in nucleated cells/kg (above median value) not only were associated with a lower TRM but also with a reduced RI, with no effect on GVHD. In high-risk patients, the Seattle group, studying transplantation with unrelated donors, made similar observations,15 that is, an increase in LFS with higher stem cell doses due mainly to a reduction in TRM but also at least in part to an enhanced graft-versus-leukemia (GVL) effect. To explain the reduced RI in relation to the higher dose of cells infused, 2 mechanisms have been proposed, a stem cell competition effect whereby an expanded normal stem cell pool might have a growth advantage over the minimal residual tumor population, and higher numbers of lymphocytes infused with the richer stem cell graft inducing more GVL, both effects possibly combining.

PB grafts contain 5 to 10 times more nucleated cells and about 10 times more T lymphocytes than BM grafts.12 PB following mobilization with granulocyte colony-stimulating factor (G-CSF) has more T polarized cells (Th2) with anti-inflammatory cytokines (hence supposedly the absence of more GVHD, as initially feared),13 and more CD14+ cells. In contrast, mesenchymal cells are present only in BM (approximately 1/10^4 BM cells/kg) and virtually absent in mobilized PB.22 Richer marrows may contain higher doses of mesenchymal stem cells.

Other types of accessory cells also may be differentially distributed in the 2 products and there may be so far unforeseen advantages in using BM, or even combining BM and blood.

Allografting with PB is associated not only with better kinetics of engraftment but also more rapid immune reconstitution.12 Despite an increase in GVHD and a questionable increase in aGVHD with PB, which would favor the use of BM, PB tends presently to be preferred by many teams, but the general assumption is that most of its benefit comes from the higher stem cell dose infused. However, in all studies comparing PB to BM the dose of BM infused never has been considered. In addition, in all studies comparing PB to BM, the median number of BM nucleated cells infused was lower than in the present study. In this respect this study may be of importance because it draws attention to the fact that infusion of rich BM during allogeneic stem cell transplantation from an HLA-identical sibling gives a better outcome than PB, whereas low-dose BM and PB are equivalent. Previous studies comparing PB to BM not only have not taken into account the doses for BM, but also have mixed several diseases and disease status. Because the present study is homogeneous in that it concerns only patients with AML in CR1, the present finding may not necessarily apply to other hematologic malignancies or more advanced stages of AML.

Appendix

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References


Hematopoietic stem cell transplantation for adults with acute promyelocytic leukemia in the ATRA era: a survey of the European Cooperative Group for Blood and Marrow Transplantation

We performed a survey of the European Cooperative Group for Blood and Marrow Transplantation to analyze the outcome of 625 acute promyelocytic leukemia (APL) patients transplanted with autologous- or allogeneic-hematopoietic stem cell transplantation (autoHCT, alloHCT) after 1993. In first (CR1) or in second complete remission (CR2), Leukemia-free survival (LFS) at 5 years in CR1 was 69% for 149 patients autografted and 68% for 144 patients allografted, whereas in CR2, LFS was 51% in 195 autoHCT and 59% in 137 alloHCT recipients, respectively. In the group of autoHCT for CR1 (n = 149), higher relapse incidence (RI) was associated with shorter time from diagnosis to transplant (<7.6 months); transplant-related mortality (TRM) was increased in older patients (<47 years), whereas for CR2, longer time from diagnosis to transplant (<18 months) was associated with increased LFS and decreased RI. In the alloHCT group for CR1 (n = 144), age (<35 years) was associated with increased LFS and decreased TRM and for CR2 (n = 137), the use of mobilized peripheral blood stem cells was associated with decreased TRM. Female recipient, a female donor to male recipient and transplants performed before 1997 were associated with decreased RI. In conclusion, HCT still appears to have a role in APL, especially for patients in CR2.

Introduction

Several large multicenter studies have shown that regimens combining upfront all-trans retinoic acid (ATRA) and chemotherapy lead to a high curability rate in acute promyelocytic leukemia (APL) that currently exceeds 70% of cases, suggesting that hematopoietic stem cell transplantation (HCT) need not be used to consolidate patients in first complete remission (CR1). In spite of this progress, however, treatment failure still occurs in approximately 10-25% of patients receiving state-of-the-art therapy due to early death or, more frequently, disease relapse.

When clinical relapse occurs, or when it is predicted accurately by molecular monitoring, HCT remains a widely adopted strategy as a part of the salvage therapy. However, there is no general consensus on the choice of transplant type, autologous (autoHCT) or allogeneic (alloHCT) in this setting. In this regard, HCT results in the ATRA era, and particularly outcome data of auto and alloHCT in CR2, have only been reported in small and non-comparative patient series. Results from these studies have suggested that autoHCT in CR2 is still associated with a high probability of long-term survival, particularly for patients undergoing the procedure while in molecular remission (i.e. testing polymerase chain reaction (PCR)-negative for the PML/RARx (promyelocytic leukemia-retinoic acid receptor z) hybrid gene pre-transplant).
In a recent analysis of the European APL group, allogeneic HSCT for 23 APL relapsed patients was associated with higher transplant-related mortality (TRM) compared with autologous HSCT. Since the last EBMT survey by Mandelli et al. in 1994, no studies in large patient series have been reported and more importantly there are no recent data available on stem cell transplantation in CR1, which is considered as a minor, if not unreasonable therapeutic option. Therefore, it is of interest to analyze the current retrospective results and risk factors of HSCT in APL to investigate further the place of autoHSCT and alloHSCT in APL (both in CR1 and in CR2) after the advent of ATRA.

Patients and methods

Patients

Data for 625 adult APL patients undergoing HSCT were reported to the Acute Leukemia Working Party of the European Cooperative Group for Blood and Marrow Transplantation (EBMT). Diagnosis of APL was based on morphological criteria according to the French-American-British classification. Results of HSCT in patients with APL transplanted before 1993 were analyzed partially by Mandelli et al. and reported in 1994. In the present study, we analyzed the results of HSCT in 625 patients in CR (293 in CR1 and 332 in CR2) transplanted between January 1993 and December 2003. In the CR1 group, 149 and 144 patients received an autoHSCT and an alloHSCT, respectively, whereas 195 and 137 patients in CR2 were autografted and allografted, respectively. For the alloHSCT group, only patients with a graft from an HLA-matched sibling were included in the study. The main clinical characteristics of the patients are reported in Table 1.

Owing to the type of study, consisting of a retrospective analysis based on information obtained from the registry, no data were available as to whether or not patients had received ATRA before stem cell transplantation (SCT). Given the considered historic period (1993–2003), however, we assume that most patients in study had been treated with ATRA and chemotherapy, as this was the strategy adopted commonly in most European hematological institutions. Similarly, no information on patient molecular status (reverse transcriptase PCR of PML/RARα) pre- and post-SCT was available.

End points definitions and statistical analysis

Data were analyzed as of 30 June 2005. Median follow-up was 42 months (range 1–132 months).

Four outcomes were studied in this series: (i) TRM was defined as all causes of non-leukemic deaths; (ii) relapse incidence (RI) was defined on the basis of morphological evidence of leukemia in bone marrow, or other extramedullary organs. To evaluate the probability of relapse, patients dying either from direct toxicity of the procedure or from any other cause not related to leukemia were censored; (iii) leukemia-free survival (LFS) was defined as time interval from transplant to first event (either relapse or death in CR); and (iv) overall survival (OS). All outcomes were calculated for CR1 and CR2 patients separately.

Patient or graft characteristics (listed in Tables 2 and 3) were analyzed for their potential prognostic value on each of the outcomes and in each type of transplant (autoHSCT or alloHSCT). For continuous variables, we used either clinically meaningful cut points (interval diagnosis to CR2 less or more than 18 months) or the median as a cut point. Statistical analyses were performed independently for each end point. The incidence of each event was non-parametrically estimated. Probability of OS and LFS were estimated by the product-limit method. The significance of differences between curves was estimated by the log-rank test (Mantel–Cox). Then, all variables were included in Cox proportional hazard model. To have a minimum follow-up period of 18 months, the analysis was performed in June 2005 on patients transplanted until December 2003.

Relapse and non-relapse mortality are mutually competing events. Accordingly, estimations of incidence of these events relied on the non-parametric estimator of cumulative incidence curves, whereas multivariate analyses were based on the proportional hazard model for this subdistribution of competing risks. These analyses were performed using the cmprsk package (developed by Gray, June 2001) on Splus 2000 and SPSS softwares.

Results

Number of HSCT in Europe from 1993 to 2003

Table 1 lists the number of HSCT (auto or allo) for patients in CR1 or CR2. The number of HSCT has decreased progressively for patients in CR1 since 1998; however for those patients transplanted in CR2, it has remained stable until 2002. In 2002 and 2003, there was also a decrease for patients transplanted in CR2.

Autotransplantation

Patients in CR1. The cumulative incidence of TRM and RI at 5 years were 10±3 and 21±4%, respectively. The 5-year estimate of LFS was 69±4% (Figure 1a). Table 2 shows the univariate analysis of variables associated with TRM, RI and LFS. In a multivariate analysis, higher RI was associated with shorter time from diagnosis to transplant (<7.6 months) (HR = 0.32, 95 CI = 0.14–0.75, P = 0.009), and TRM was increased in older patients (>47 years) (HR = 3.7, 95 CI = 1.3–10.5, P = 0.045) (Figure 1b). No risk factor was selected in the model for LFS.

Patients in CR2. For 195 patients transplanted in CR2, the 5-year cumulative incidence of TRM was 16±3% and the RI was 37±4%. LFS at 5 years was 51±4% (Figure 1c). In a univariate analysis, the 5-year estimates of RI and LFS were 31±4 and 56±5% for patients transplanted 18 months after diagnosis compared respectively with 25±7 and 53±6% for those transplanted earlier (Table 2). In a multivariate analysis, only longer time from diagnosis to HSCT (>18 months) was associated with increased LFS (HR = 0.43, 95 CI = 0.27–0.65, P < 0.0001) and decreased RI (HR = 0.42, 95 CI = 0.25–0.71).
Table 1  Characteristics of adult patients with APL given an auto- or alloHSCT according to disease status at transplantation

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<th>AutoHSCT CR1 (n = 149)</th>
<th>AutoHSCT CR2 (n = 185)</th>
<th>AlloHSCT CR1 (n = 144)</th>
<th>AlloHSCT CR2 (n = 137)</th>
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<td>33 (25)</td>
<td>25 (38)</td>
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<td>Purge (%)</td>
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<td>22 (14)</td>
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<td>Interval from diagnosis to CR1 (days)</td>
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<td>87</td>
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<tr>
<td>CSA + others (%)</td>
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<td>32</td>
<td>13</td>
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<td>Conditioning</td>
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<td>TBI associated (%)</td>
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<td>54 (39)</td>
<td>64 (48)</td>
<td>70 (53)</td>
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</table>

Abbreviations: APL = acute promyelocytic leukemia; BM = bone marrow; CR1 = first complete remission; CR2 = second complete remission; CSA = cyclosporin A; HSC = hematopoietic stem cell; HSCT = hematopoietic stem cell transplantation; MTX = methotrexate; PB = peripheral blood; PBSC = peripheral blood stem cells; SC = stem cells; SCT = stem cell transplantation; TBI = total body irradiation.

P = 0.001). No statistically significant risk factor was found to be associated with TRM.

Allogeneic HSCT

Patients in CR1. Acute GVHD II–IV was observed in 30% of the patients (32 patients had grade II, eight grade III and five grade IV) and chronic GVHD was observed in 36% of patients at risk. The 5-year estimates of TRM, RI and LFS for patients were 20.4%, 12.4% and 68.4% (Figure 2a), respectively. Table 3 lists the univariate analysis for 5-year outcomes.

The 5-year estimates of cumulative incidence of TRM and LFS for patients younger than 33 years were 10.4% (Figure 2b) and 78.5%, respectively. There was a trend of lower TRM for patients allotransplanted more than 6 months after diagnosis (Table 3). In a multivariate analysis, recipient age (>33 years) was associated with decreased LFS (HR = 2.25, 95 CI = 1.20–4.20, P = 0.01) and higher TRM (HR = 3.32, 95 CI = 1.38–8.00, P = 0.007). A longer time from diagnosis to HSCT (>6 months) was also an independent factor associated with lower TRM (HR = 0.46, 95 CI = 0.22–0.95, P = 0.036). No risk factor studied was associated with the RI in this setting.

Patients in CR2. Acute GVHD II–IV was observed in 35% of patients (28 patients had grade II, 10 grade III and 11 grade IV) and chronic GVHD was observed in 39% of patients at risk. The 5-year estimate of LFS was 59.4% (Figure 2c). Table 3 lists the univariate analysis of LFS, TRM and RI at 5 years.

Bone Marrow Transplantation
Table 2  Univariate analysis of 5 years LFS, RI and TRM for patients with APL given an autoHSCT according to disease status at transplant

<table>
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<tr>
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<td></td>
<td>N</td>
<td>LFS</td>
</tr>
<tr>
<td>Overall</td>
<td>149</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>Age</td>
<td></td>
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<tr>
<td>&lt; median</td>
<td>74</td>
<td>73 ± 6</td>
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<tr>
<td>P-value</td>
<td>0.89</td>
<td>0.09</td>
</tr>
<tr>
<td>&gt; Median</td>
<td>75</td>
<td>64 ± 6</td>
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<tr>
<td>Patient sex</td>
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</tr>
<tr>
<td>Male</td>
<td>83</td>
<td>69 ± 6</td>
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<tr>
<td>Female</td>
<td>66</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>P-value</td>
<td>0.89</td>
<td>0.18</td>
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<tr>
<td>TBI</td>
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<tr>
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<td>67 ± 8</td>
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<td>P-value</td>
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<td>Source of HSC</td>
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<td>BM</td>
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<td>PB</td>
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<td>P-value</td>
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<td>0.23</td>
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<td>Interval from diagnosis to transplantation</td>
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<td>&lt; 6 months</td>
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<td>P-value</td>
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<td>&gt; 6 months</td>
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<td>60 ± 8</td>
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<td>P-value</td>
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<td>Interval from diagnosis to transplantation</td>
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<tr>
<td>&lt; 8 months</td>
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<td>P-value</td>
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<td>Year of transplantation</td>
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<td>&lt; median</td>
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<td>P-value</td>
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<td>&gt; Median</td>
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<td>WBC at diagnosis</td>
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<tr>
<td>&gt; Median</td>
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<td>Purge</td>
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<td>No</td>
<td>139</td>
<td>47 ± 6</td>
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<td>P-value</td>
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Abbreviations: APL = acute promyelocytic leukemia; BM = bone marrow; CR1 = first complete remission; CR2 = second complete remission; HSC = hematopoeitic stem cell transplantation; LFS = leukemia-free survival; RI = relapse incidence; TRM = total body irradiation; TRM = transplant-related mortality; PB = peripheral blood; WBC = white blood cells.

The symbol ± denotes the standard error.

Cumulative incidence of TRM at 5 years was 24 ± 1%. In univariate analysis, cumulative incidence of TRM was 12 ± 4% for allotransplants using peripheral blood stem cells (PBSC) and 31 ± 4% for those using bone marrow cells as a source of hematopoietic stem cells (P = 0.008). There was also significant statistical association of TRM with the period of transplant, that is 15 ± 4% for patients transplanted after 1997 compared with 31 ± 5% for those transplanted before this period (P = 0.03). In a multivariate analysis for TRM, only PBSC transplants were associated with lower TRM (HR = 0.30, 95 CI = 0.12-0.77, P = 0.03).

Cumulative incidence of relapse at 5 years was 17 ± 1%. Results of univariate analysis are listed in Table 3. High white blood cells (WBC) (> median) at diagnosis were a
Table 3  Univariate analysis of 5 years LFS, RI and TRM for patients with APL given an allogHCT according to disease status

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<td>RI</td>
<td>TRM</td>
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<td>N</td>
<td>LFS</td>
<td>RI</td>
<td>TRM</td>
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Abbreviations: APL = acute promyelocytic leukemia; BM = bone marrow; CRI1 = first complete remission; CRI2 = second complete remission; HSC = hematopoietic stem cell; HCT = hematopoietic stem cell transplantation; LFS = leukemia-free survival; RI = relapse incidence; TBI = total body irradiation; TRM = transplant-related mortality; PB = peripheral blood; WBC = white blood cells.

The symbol ± denotes the standard error.
Figure 1: Outcome of patients autografted: (a) LFS in patients autografted in CR1; (b) cumulative incidence of TRM according to age in patients autografted in CR1; and (c) LFS in patients autografted in CR2.

Figure 2: Outcome of patients allografted: (a) LFS in patients allografted in CR1; (b) cumulative incidence of TRM according to age in patients allografted in CR1; and (c) LFS in patients allografted in CR2.

statistically significant factor ($P=0.008$) associated with higher relapse rate (Table 3). In a multivariate analysis, the following factors were associated with decreased incidence of relapse: (i) transplants performed before 1997 (HR = 4.8, 95% CI = 1.8–13, $P=0.002$), (ii) female recipient (HR = 0.30, 95% CI = 0.10–0.84, $P=0.02$) and (iii) female donor to male recipient (HR = 0.14, 95% CI = 0.03–0.55, $P=0.003$). High WBC count was not included in the model due to absence of relapse for those patients with lower WBC count at diagnosis.

Discussion

This survey of the EBMT Group shows that HSCT activity in Europe has decreased progressively since 1998 for...
patients with APL in CR1, whereas the number of patients transplanted in CR2 has remained stable over the study period. In addition, the data from this survey confirms a surprisingly low LFS in allo- and autoHSCT groups, as well as a lower TRM and a higher RI in autoHSCT than in alloHSCT. According to the type of transplant, we were also able to find certain risk factors for outcomes such as age, time from diagnosis to transplant, year of transplants, source of stem cells and donor gender.

The first objective of our study was to perform a survey on the results of HSCT for APL patients in the ATRA era. To our knowledge, no studies including large series of patients with APL receiving an auto- or alloHSCT have been reported after the first EBMT survey published in 1994 by Mandelli et al. when ATRA was not yet used in the majority of the European centers. When we planned the present study, we did not expect to discover such a high number of patients with APL still transplanted after 1993, a number equivalent to those transplanted before 1993 (over a similar period of time), which at that time included mostly patients treated with only chemotherapy.

Although we were unfortunately unable to obtain complete data relative to pre-transplant treatments in the registry, it is conceivable that most patients here analyzed had received modern ATRA-containing regimens pre-HSCT. In fact, the present study included patients transplanted after 1993, when front-line ATRA therapy was adopted for both newly diagnosed and relapsed APL in most European Centers. However, we have observed that the number of patients transplanted in CR1 with an auto- or alloHSCT has decreased progressively since 1998, whereas the number of patients transplanted in CR2 has remained quite stable but with a decrease in 2003, probably reflecting the adoption for these patients of other therapeutic strategies such as arsenic trioxide (ATO). Although the justification for transplanting patients in CR2 is quite obvious, it is unclear why patients with APL were transplanted in CR1. However, those transplants were performed mostly during the earlier years after the introduction of ATRA-based regimens, whereas only few patients in CR1 are currently transplanted, in particular those showing persistent molecular disease after front-line consolidation.

Compared with the previous EBMT survey, the present EBMT study shows a higher LFS, confirming a quite similar final outcome in allo- and autoHSCT groups. This similar final outcome seems related to a significant reduction of TRM in the last years (8% in autoHSCT and 17% in alloHSCT) and to the counterbalance between the higher RI and TRM rates observed in the auto- and alloHSCT groups, respectively. In addition to some improvement over time, these better results may be related to the wide use of ATRA pre- and/or post-transplant.

Our second objective was to analyze risk factors for outcomes, mainly TRM, RI and LFS for patients receiving either auto- or alloHSCT in CR1 and most importantly in CR2. As expected, in first CR, patient's age, that is younger than 33-years old, was the most important factor associated with decreased TRM in both auto- and alloHSCT settings.

Therefore, the option of alloHSCT should be considered seriously for younger patients in CR1 with persistent molecular disease after consolidation, whereas for older patients who test PCR-positive at this time point, other treatment options, such as ATO or gemtuzumab-ozogamicin may be considered.

In CR2, both approaches also produced reasonably good results. In the autoHSCT setting, LFS at 5 years was 51% for the entire study period and more than 60% after 1997. Importantly, patients with longer CR1 duration (diagnosis to transplant >18 months) had a better LFS and a lower RI. This observation makes sense because patients relapsing early are more likely to having received autografts still contaminated by tumor cells. Indeed autografting is recommended presently in APL patients in CR2 only if disappearance of minimal residual disease by PCR has been documented. Nevertheless, some investigators have proposed that the role of autoHSCT to consolidate high-risk patients (i.e. with hyperleucocytosis) should be investigated. Unfortunately, in our retrospective study, which was based on data from a registry, no information was available about the PML/RARA molecular status of the graft and/or of patient bone marrow at transplant. In addition, other relevant information was neither available, including the type of previous front-line nor salvage therapy used, the reason for the indication of transplant in CR1, as well as the type of transplant, among others.

For APL patients with an HLA-identical sibling, alloHSCT continues to be recommended in most centers as the treatment of choice for patients in CR2. We have found that the use of PBSC was associated with decreased TRM as compared to bone marrow. This finding is in agreement with the results of randomized studies comparing bone marrow and PBSC transplants, which showed better results for PBSC in patients transplanted with more advanced disease. Interestingly, decreased RI was associated with a female donor to male recipient suggesting a graft-versus-leukemia effect mediated probably by minor H-Y antigens. Of note, we have observed that a higher WBC count at diagnosis was also an important risk factor for increased RI after autotransplantation. Another interesting finding is that post-transplant RI has increased after 1997, probably reflecting a selection of patients with more aggressive disease relapsing after receiving state-of-the-art front-line regimens.

In conclusion, the present study indicates that HSCT has been decreasing over the years for patients with APL in CR1 in Europe, but it has continued to be part of the treatment strategy for patients in CR2. The data from this survey indicates that, in spite of the favorable results with both auto- and alloHSCT (LFS around 70% at 5 years), HSCT should no longer be used to consolidate CR1 in patients with APL treated with modern ATRA plus chemotherapy regimens. Only selected and young patients with persistent molecular disease can probably benefit from this approach. Likewise, our results show that a high proportion of patients in CR2 achieve long-term OS after auto- and alloHSCT, and both procedures represent valid therapeutic options in this setting. The choice of one or other procedure will depend on the availability of an HLA
identical donor and the time from diagnosis to transplant. Finally, in the near future, HSCT results will need to be compared with long-term results of other therapeutic options for APL relapse such as ATO and gemtuzumab ozogamicin, which have provided promising outcome data in small series with limited follow-up.

References


Combination Chemotherapy Versus Melphalan Plus Prednisone as Treatment for Multiple Myeloma: An Overview of 6,633 Patients From 27 Randomized Trials

By the Myeloma Trialists' Collaborative Group

Purpose: To compare combination chemotherapy (CCT) versus melphalan plus prednisone (MP) as treatment for multiple myeloma.

Patients and Methods: In a collaborative worldwide overview of randomized trials of CCT versus MP, individual patient data on 4,930 patients from 20 trials were analyzed, with the addition of published data on a further 1,703 patients from seven trials. The main outcome measure was mortality, with response and recurrence rates being subsidiary end points.

Results: Taking all of the trials together, response rates were significantly higher with CCT than with MP (60.0% v 53.2%; P < .00001, two-tailed). There was no evidence of any difference in mortality between CCT and MP, and with a nonsignificant 1.5% reduction in death rate in favor of CCT (P = .6, two-tailed). There is heterogeneity of design between the trials, but subgroup analyses by type of CCT or by dose-intensities of CCT, of melphalan, or of prednisone did not identify any particular forms of therapy that were either clearly beneficial or clearly adverse. Similarly, analysis of the presentation features of the patients did not find any categories in which CCT differed significantly from MP in its effects on mortality; in particular, there was no evidence that poor-risk patients benefited more from CCT.

Conclusion: This overview found no difference, either overall or within any subgroup, in mortality between CCT and MP. In terms of survival, those therapeutic options, as tested in the trials considered, are approximately equivalent.


For many years, the standard induction chemotherapy for patients with multiple myeloma has been melphalan and prednisone (MP). In an attempt to improve the outcome of these patients, many randomized trials have compared MP with combination chemotherapy (CCT) regimens that contain three or more drugs. A meta-analysis of the published data from some of these trials has been performed, but such reviews of the published literature have important limitations. The Myeloma Trialists' Collaborative Group therefore sought to increase the reliability of the evidence on the comparative efficacy of these two treatments by performing an overview using the individual patient data from all of the randomized trials of MP versus CCT.

Patients and Methods

Trials in which patients were randomly allocated to CCT versus MP were searched for extensively by computer-aided search of publications and clinical trial data bases, with search of meeting abstracts, by scrutiny of reference lists of trials and review articles, and by communication with trialists and pharmaceutical companies. This was done as part of a wider overview of all treatments for myeloma. A limited amount of data was sought for each patient randomized in all relevant trials that began before 1990. Trials were excluded if they were found not to be properly randomized—for example, if the allocation method allowed foreknowledge of the treatment that would be assigned if the patient were to be entered onto the trial (eg, date of birth, day of week of entry).

The diagnostic data requested for each randomized patient were as follows: patient identifier, date of diagnosis, date of birth (or age at randomization), sex, Durie-Salmon stage, hemoglobin level, platelet count, WBC count, β2-microglobulin level, M-band type, creatinine concentration, calcium and albumin levels, presence of bone lesions, and performance status. The event data requested were as follows: date randomized, allocated treatment, type of response (complete, partial) and date of response, achievement date of plateau phase, recurrence and its date, current status (alive, dead), and date of death or date of last follow-up evaluation. Data were checked for consistency with any publications, for internal inconsistencies, for balance between treatment groups and for the exclusion of randomized, or the inclusion of nonrandomized, patients. Any apparent problems were referred to the responsible trialists for clarification, so that errors and omissions could be rectified as fast as possible. Summary tables of the results for each trial used in the overview were supplied to the trialists for checking, and a draft of the present report was circulated to each trial group for their comments and to ensure that the results used for their trial were correct.

Standard statistical methods were used. Observed minus expected (O−E) number of events in each treatment group was calculated, and its variance (V) was calculated for each separate trial by means of log-rank survival analysis using the exact dates of any events. These quantities, one per
Trial, are then summarized for all trials to give two grand totals. This ensures that patients within a trial are directly compared only with other patients in the same trial, and not with those in other trials. The grand totals are then used to calculate odds ratios (OR), odds reductions (where an OR of 0.99 would correspond to an odds reduction of 10%) and their 95% confidence intervals (CIs). Tests of heterogeneity or trend are used to examine differences in treatment effect between trials and between different subgroups of patients. However, such tests of heterogeneity are statistically insensitive and so their results must be interpreted cautiously.

The few trials from which individual patient data were not available (Table 1) are not included in the survival curves, since time to event was unknown and it was not possible to perform log-rank survival analyses. Moreover, since information on prognostic factors, or on end points

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Table 1. Trials of CCT Versus MP
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<td>SWOG 770111.17</td>
<td>1977-79</td>
<td>3-wk cycle: Pred 60 mg/m² d1-d4, Mel 8 mg/m² d1-d4</td>
<td>To 6-12m, then reduces after 12m</td>
<td>3.1</td>
<td>3.7</td>
<td>10.7</td>
</tr>
<tr>
<td>77E</td>
<td>MOA 770807</td>
<td>1977-80</td>
<td>25-d cycle: Pred 100 mg d1-d4, Mel 7 mg/m² d1-d4</td>
<td>To 6 then all received 25-d cycles of VMCP as in induction, for 12m with randomization between lenalidomide or no lenalidomide</td>
<td>2.9</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td>77F</td>
<td>MMASG MA-77F</td>
<td>1977-80</td>
<td>Monthly: Pred 60 mg/m² d1-d8, Mel 0.1 mg/m² d1-d7</td>
<td>To 6 then excessive; if &gt; 75% reduction in tumor; if 50% to 75% reduction, continue for 12m</td>
<td>2.9</td>
<td>2.5</td>
<td>7.0</td>
</tr>
<tr>
<td>77G</td>
<td>Panico MA-77G</td>
<td>1977-84</td>
<td>2-wk cycle: Pred 0.5 mg/kg d1-d5, Mel 0.25 mg/m² d1-d4</td>
<td>After 3 cycles, patients switched to MP; all received lenalidomide continued until relapse</td>
<td>2.3</td>
<td>2.3</td>
<td>6.7</td>
</tr>
<tr>
<td>79B</td>
<td>BCOG 24752</td>
<td>1979-83</td>
<td>4-wk cycle: Pred 40 mg/m² d1-d4, Mel 8 mg/m² d1-d4</td>
<td>To 30m then for initial response: d1: treated if residual paraprotein; d1-d7 or all continued every 4 wk x 3 then every 8 wk to relapse</td>
<td>2.5</td>
<td>2.8</td>
<td>8.0</td>
</tr>
<tr>
<td>79J</td>
<td>Gentofte, Denmark</td>
<td>1979-83</td>
<td>4-wk cycle: Pred 0.5 mg/kg d1-d4 then 0.3 mg/kg d1-d7 per cycle, Mel 0.1 mg/kg d1-d7, BONU 0.5 mg/kg d1, Vinc 0.03 mg/kg d1</td>
<td>To 30m then for initial response: d1: treated if residual paraprotein; d1-d7 or all continued every 4 wk x 3 then every 8 wk to relapse</td>
<td>2.9</td>
<td>3.0</td>
<td>10.5</td>
</tr>
<tr>
<td>80A</td>
<td>NARC MYEL-425</td>
<td>1980-82</td>
<td>4-wk cycle: Pred 40 mg/m² d1-d7, Mel 10 mg/m² d1-d7, Vinc 1 mg d1</td>
<td>To plateau phase then randomized between no further treatment or continue for 4y</td>
<td>3.6</td>
<td>3.0</td>
<td>10.5</td>
</tr>
<tr>
<td>80C</td>
<td>Finnish MA-80C</td>
<td>1980-82</td>
<td>3-wk cycle: Pred 1.0 mg/kg d1-d7, Mel 0.1 mg/kg d1-d7</td>
<td>To plateau phase then randomized between no further treatment or continue for 4y</td>
<td>3.3</td>
<td>3.0</td>
<td>10.0</td>
</tr>
<tr>
<td>80E</td>
<td>RANSK MA-80</td>
<td>1980-82</td>
<td>3-wk cycle: Pred 60 mg/m² d1-d4, Mel 6 mg/m² d1-d4, BONU 10 mg/m² d1, Vinc 1 mg d1</td>
<td>To 24m</td>
<td>3.4</td>
<td>3.1</td>
<td>10.5</td>
</tr>
<tr>
<td>81B</td>
<td>Hornstad, Norway</td>
<td>1981-82</td>
<td>3-wk cycle: Pred 100-150 mg [male weight] d1-d4, Mel 0.25 mg/kg d1-d4, BONU 0.5 mg/kg d1</td>
<td>To ly then randomized to stop or continue with 10-wk cycles</td>
<td>3.7</td>
<td>2.3</td>
<td>6.7</td>
</tr>
<tr>
<td>Year Code</td>
<td>Trial Name</td>
<td>Entry Period</td>
<td>CCT Arm</td>
<td>MP (or similar) Arm</td>
<td>Duration of Therapy</td>
<td>Dose Intensity*</td>
<td>MP Dose (mg/m²/ wk)</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>--------------</td>
<td>---------</td>
<td>---------------------</td>
<td>---------------------</td>
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</tr>
<tr>
<td>81E</td>
<td>MGCS stage I-II</td>
<td>1982-83</td>
<td>VMCP (3-wk cycles to response then 6-wk cycles); Vinc 1 mg/d1, Mel 4 mg/m² d1-d4, Cyclo 100 mg/m² d1-d4, Pred 60 mg/m² d1-d3 alternating with VBAP (3-wk cycles to response then 6-wk cycles); Vinc 1 mg/d1, BOCNU 30 mg/m² d1, Adri 30 mg/m² d1, Pred 60 mg/m² d1-d4</td>
<td>6-wk cycle: Pred 2 mg/kg d1-d4, Mel 0.25 mg/kg d1-d4</td>
<td>To progression or relapse</td>
<td>4.5</td>
<td>2.3</td>
</tr>
<tr>
<td>82E</td>
<td>GWMFS MAB23</td>
<td>1982-83</td>
<td>4-wk cycle; Pred 60 mg/m² d1-d4, Mel 5 mg/m² d1-d4, Cyclo 100 mg/m² d1-d4, Vinc 1 mg d1</td>
<td>4-wk cycle; Pred 60 mg/m² d1-d4, Mel 8 mg/m² d1-d4</td>
<td>To 24 wk, then randomized to stop or continue every 4 wk until relapse</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>83E</td>
<td>NAMSG M-83</td>
<td>1983-86</td>
<td>VMCP (28-d cycle); Vinc 1 mg d1, Mel 6 mg/m² d1-d7, Cyclo 120 mg/m² d1-d7, Pred 60 mg/m² d1-d7 alternating with VBAP (28-d cycle); Vinc 1 mg d1, BOCNU 30 mg/m² d1, Adri 30 mg/m² d1-d7, Pred 60 mg/m² d1-d7</td>
<td>4-wk cycle; Pred 2 mg/kg d1-d4, Mel 0.25 mg/kg d1-d4</td>
<td>To 12 m in plateau, otherwise until relapse or death</td>
<td>5.1</td>
<td>3.9</td>
</tr>
<tr>
<td>83H</td>
<td>MGWS stage I-II</td>
<td>1983-86</td>
<td>VMCP (4-wk cycles); Vinc 1 mg d1, Mel 5 mg/m² d1-d4, Cyclo 100 mg/m² d1-d4, Pred 60 mg/m² d1-d4</td>
<td>4-wk cycle; Pred 2 mg/kg d1-d4, Mel 0.25 mg/kg d1-d4</td>
<td>To 12 m in plateau, otherwise until relapse or death</td>
<td>2.9</td>
<td>2.3</td>
</tr>
<tr>
<td>83I</td>
<td>MGWS stage I-II</td>
<td>1983-86</td>
<td>VMCP (4-wk cycles); Vinc 1 mg d1, Mel 5 mg/m² d1-d4, Cyclo 100 mg/m² d1-d4, Pred 60 mg/m² d1-d4</td>
<td>4-wk cycle; Pred 2 mg/kg d1-d4, Mel 0.25 mg/kg d1-d4</td>
<td>To 12 m in plateau, otherwise until relapse or death</td>
<td>3.5</td>
<td>2.3</td>
</tr>
<tr>
<td>83C</td>
<td>PETHEMA 85</td>
<td>1985-89</td>
<td>VMCP (4-wk cycles); Vinc 1 mg d1, Mel 6 mg/m² d1-d4, Cyclo 500 mg/m² d1-d4, Pred 60 mg/m² d1-d4 alternating with VBAP (4-wk cycle); Vinc 1 mg d1, BOCNU 30 mg/m² d1, Adri 30 mg/m² d1, Pred 60 mg/m² d1-d4</td>
<td>4-wk cycle; Pred 60 mg/m² d1-d4, Mel 9 mg/m² d1-d4</td>
<td>To 32 wk, if responders receive further 22-wk treatment after which objective responders ceased treatment</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>86G</td>
<td>Pavia 88</td>
<td>1986-89</td>
<td>Vinc 0.4 mg/kg d1-d7, Pred 0.5 mg/kg d1-d7, Mel 0.21 mg/kg d1-d7, Vinc 0.025 mg/kg d1-d4</td>
<td>6 x 6-wk cycles: Pred 0.5 mg/kg d1-d10, Mel 0.21 mg/kg d1-d4</td>
<td>All responders randomized between continue to plateau or to relapse</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>86I</td>
<td>GEM 88</td>
<td>1987-89</td>
<td>No details available</td>
<td>No details available</td>
<td>No details available</td>
<td>4-wk cycle; Pred 100-200 mg by weight d1-d14, Mel 0.25 mg/m² d1-d14</td>
<td>4-wk cycle; Pred 60 mg/m² d1-d4, Mel 15 mg/m² d1-d4</td>
</tr>
<tr>
<td>86D</td>
<td>GWMFS MAB23</td>
<td>1989-91</td>
<td>Devam 25 mg/m² IV d1-d7/d2-4/wk, Pred 40 mg IV d1-d7/d2-4/wk, Vinc 7 mg/m² d1-d7/d2-4, Adri 15 mg/m² d1-d7/d2-4, BOCNU 40 mg/m² d1-d7/d2-4</td>
<td>4-wk cycle; Pred 60 mg/m² d1-d4, Mel 15 mg/m² d1-d4</td>
<td>To maximum response, then randomized for no further treatment w/ weekly infusion</td>
<td>8.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Other CCTs:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>83F</td>
<td>Nagoya 83</td>
<td>1985-87</td>
<td>3-wk cycle; Vinc 1 mg/m² d1-d4, Pred 30 mg/m² d1-d4, Cyclo 100 mg/m² d1-d4, Pred 30 mg/m² d1-d4</td>
<td>Pred (30 mg/m² d1-d4) from 7 mg/m² d2-4 d1-d4, Pred 30 mg/m² d1-d4</td>
<td>To plateau, then continued for 2 yr or longer intervals</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>82F</td>
<td>MRC MYEL I-5</td>
<td>1982-86</td>
<td>ABDM (4-wk cycle); Adri 30 mg/m² d1, BOCNU 30 mg/m² d1, Cyclo 100 mg/m² d1-d4, Pred 60 mg/m² d1-d4</td>
<td>3-wk cycle; Mel 7 mg/m² d1-d4</td>
<td>To plateau or relapse</td>
<td>3.4</td>
<td>2.3</td>
</tr>
</tbody>
</table>

NOTE: Adr, BOCNU, Mitox, Pep, and Vinc were always administered IV. For other drugs, if the route is not stated, the preparation was administered PO. Trials for which IPD were not available are indicated by parentheses or brackets around the year code; parentheses indicate that published data were used, brackets indicate that no data were available.

Abbreviations: SWOG, Southwest Oncology Group; CALGB, Cancer and Leukemia Group B; ECOG, Eastern Cooperative Oncology Group; SECOG, Southeastern Cancer Study Group; GATSA, Grupo Argentina de Tratamiento de la Leucemia Aguda; NCIC, National Cancer Institute of Canada; WCG, Western Cancer Study Group; MDA, MD Anderson Hospital and Tumor Institute; NAMSG, Italian Multiple Myeloma Study Group; MRC, Medical Research Council; MGCS, Myeloma Group of Central Sweden; PETHEMA, German Myeloma Treatment Group; GWMFS, Myeloma Group of Western Sweden; GEM, Group d'Etude des Myelomes; NAMSG, Nordic Myeloma Study Group; Pred, prednisone; Mel, melphalan; Pab, procarbazine; BOCNU, vincristine; Cyclo, cyclophosphamide; MeCCNU, methyl-CCNU; Vinc, vincristine; Adr, doxorubicin; Pep, prednimustine; Bexx, dexamethasone; wk, week; d, day; m, month; y, year; IV, intravenous; PO, oral.

*See Patients and Methods for details on dose-intensity.

†Doses were adjusted to cause a degree of cytopenia.

§After 57 patients, initial melphalan doses were reduced by 25% to be increased if tolerated.
other than death, was also not generally available from these trials, it has not been possible to use them in any of the stratified analyses or in analyses of subsidiary end points. Instead, where possible, tabular data on the number of patients and deaths in each arm have been obtained from publications and are used solely in the overall analyses of mortality.

The main analyses presented are of mortality from the date of randomization, either unstratified or stratified by various prognostic factors. In addition to mortality, subsidiary analyses of response rates (complete and/or partial) and recurrence were performed for trials with individual patient data. The criteria for response for each trial were those used by the group responsible for the trial.

Where sufficient information was available, dose-intensities were calculated for the CCT and MP regimens in each trial (Table 1), using a method similar to that of Heynrick and Buth.

Calculation of Dose-Intensities

As in the published data meta-analysis by Gregory et al., the Southwest Oncology Group (SWOG) 7704/7705 regimen of vincristine, melphalan, cyclophosphamide, and prednisone (VMP)/vincristine/carmustine (BCNU), doxorubicin, and prednisone (VBAP) was used as the standard for CCT. Therefore, standard dose-intensities for individual drugs were considered to be as follows: doxorubicin 5 mg/m²/wk, BCNU 5 mg/m²/wk, cyclophosphamide 167 mg/m²/wk, lomustine (CCNU) 12.5 mg/m²/wk, melphalan 4 mg/m²/wk, prednisone (or prednisolone) 80 mg/m²/wk, and vincristine 0.5 mg/m²/wk. Dexamethasone was assumed to have an intensity of 6.7 times that of prednisone, and methylprednisolone to have an intensity of 1.25 times that of prednisone (British National Formulary, 32, 1996). For each drug, the dose-intensity was calculated in milligrams per square meter per week by calculating the total dose given over the first 24 weeks and dividing this by 24. These values were compared with the standard dose-intensities described above. Each drug was assumed to be equally efficacious, so each standard dose was given a value of 1 unit per week. Overall standard dose-intensities for both the CCT and MP regimens were calculated by summing the intensities for each drug. The route of administration for drugs such as cyclophosphamide and melphalan was assumed not to affect the intensity of the treatment.

RESULTS

Trials and Patients

Thirty trials that compared CCT versus MP were identified, involving 6,633 patients. Details, including drug dosages, duration, method of administration, and timing, are given in Table 1, in chronologic order of starting date, with each trial being given a unique reference that consists of the start year plus an arbitrary character (eg, 84A, 86D). Individual patient data were supplied from 20 of the trials (4,930 patients). Published data were abstracted for seven of...
Fig 1. Mortality in trials of CCT v MP. Large squares indicate larger trials that provide more information, and hence have narrower 95% CI. If the square is to the left of the solid line, then survival is better in the group allocated CCT, but if the CI crosses this line, then this result is not statistically significant at P < .01 (2-tailed). Open boxes indicate trials for which only published data were used. Totals are represented as diamond centroids on the OR estimate, with 95% CI shown by the width of the diamond and with the odds ratio also given as a percentage along with its standard deviation (SD). Two totals are shown, 1 for the individual patient data (IPD) and 1 including published data, with similar odds reductions in both cases.

the remainder (1,703 patients), which left only three trials with no data available. Table 2 lists the presentation features of the 4,930 patients with individual data supplied. Continuous variables (eg, hemoglobin, platelets) were grouped (to some extent with arbitrary cutoffs) into a small number of categories for the purpose of analysis.

Overall Survival

Figure 1 shows the mortality results of each trial and Fig 2 shows, for those trials that provided individual patient data, the survival curve. Overall, there is no significant difference in survival between patients allocated to CCT or MP (P = .6, two-tailed). The point estimate for the proportional reduction in the annual odds of death is 1.5% in favor of CCT, but the 95% CI is 0.92 to 2.1%, which is statistically significant at P = .01.

1.04) and those for which published data were used (OR, 1.03; 95% CI, 0.83 to 1.25; test for interaction: \( \chi^2 = 0.2, P = .7 \)).

There is clearly considerable heterogeneity between the therapies tested within the trials: the drugs and their doses within the CCT regimens vary greatly, and the doses of melphalan and prednisone within the MP regimens also differ between trials. Furthermore, the patient populations recruited to the various trials will have differed (eg, some trials included younger patients and other trials older ones; and some trials excluded stage 1 patients). Hence, within the overall null result there might be particular types of CCT that are either more or less effective, particular MP regimens that are better than others, or specific subgroups of patients for whom either CCT or MP is preferable.

To assess possible differences between CCT regimens, the trials were subdivided in various ways (Fig 3). Cut-off points between higher and lower dose categories were selected so that about half the trials were in each group. The cut points were: CCT dose-intensity, < 4.0 vs 4.0; melphalan dose,
come, e.g., increasing age, higher Durie-Salmon stage, low hemoglobin level, high creatinine concentration, and high β₂-microglobulin level. Relatively complete information was provided on all of these except the latter. Table 2 lists the survival of the individual data patient population by the various prognostic factors. Subgroup analyses of the trials by these prognostic factors shows no reliable evidence that, for any parameter, there is a group of patients that benefited more, or less, when allocated to CCT (Fig 4). In particular, there is no evidence to indicate that high-risk patients benefit from CCT.

In Fig 2, it appears that CCT is associated with a slight disadvantage during the first year and a slight advantage thereafter. Formal analyses of mortality by time period show a nonsignificant adverse effect of CCT in the first year after randomization (O - E = 28, V = 303; \( P = .1 \), two-tailed) and a nonsignificant benefit for CCT after the first year (O - E = -44, V = 618; \( P = .07 \), two-tailed). This leads to a conventionally significant interaction between the results in these two time periods (\( P = .02 \)), but this data-derived comparison is not extreme enough to demonstrate any real difference between the shapes of the survival curves.

Table 3. Response Rates (complete plus partial) in Trials of CCT Versus MP Calculated From Supplied Individual Patient Data

<table>
<thead>
<tr>
<th>Study Short Year, Code, and Name</th>
<th>Response Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCT</td>
<td>MP</td>
</tr>
<tr>
<td>72G ECOG 4572</td>
<td>60.9</td>
</tr>
<tr>
<td>72D SIEG 343</td>
<td>39.2</td>
</tr>
<tr>
<td>72B GIATA 10M-73</td>
<td>64.7</td>
</tr>
<tr>
<td>77B GIATA 3-M-77</td>
<td>64.7</td>
</tr>
<tr>
<td>77C SWOG 7704*</td>
<td>63.3</td>
</tr>
<tr>
<td>77J Pavia MM-75</td>
<td>72.2</td>
</tr>
<tr>
<td>79B ECOG 2479</td>
<td>67.2</td>
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<tr>
<td>80A MBB MIEL-41</td>
<td>41.3</td>
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<tr>
<td>80D Povil MM-90</td>
<td>70.3</td>
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<td>80E WAMSG M-80</td>
<td>50.8</td>
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<tr>
<td>81B Harstad</td>
<td>70.5</td>
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<tr>
<td>81E MCOCS 1981 (stage III)</td>
<td>52.4</td>
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<tr>
<td>82E GMG MM-01</td>
<td>56.3</td>
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<tr>
<td>83E WAMSG M-83</td>
<td>77.7</td>
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<td>83H MGWS (stage II)</td>
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<td>83I MGWS (stage III)</td>
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<td>85C FEHIANA 1985</td>
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<tr>
<td>84G Pavia 1986b</td>
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<tr>
<td>87G WTMG</td>
<td>74.0</td>
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<tr>
<td>88D GMG MM-02</td>
<td>66.7</td>
</tr>
</tbody>
</table>

Overall 60.0 53.2

NOTE: Difference in response rate. 6.8%; \( P < .00001 \).
*For balance, in this trial with a 2:1 randomization ratio, responses and patients in the MP arm are counted twice when calculating the overall response rate on MP.
†Response data not available, so percent of patients reaching plateau phase used.
OVERVIEW OF INDUCTION THERAPY FOR MYELOMA

<table>
<thead>
<tr>
<th>Trial grouping</th>
<th>Number of trials</th>
<th>Deaths/Patients</th>
<th>Statistics</th>
<th>O.R. &amp; 95% Cl (CCT: MP)</th>
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<tbody>
<tr>
<td>Type of CCT:</td>
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</tr>
<tr>
<td>VMCP + VBAP/VCPAP</td>
<td>6</td>
<td>600/786</td>
<td>240.7</td>
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<td>Other anthrapyline</td>
<td>3</td>
<td>322/1340</td>
<td>66.1</td>
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<tr>
<td>VMP</td>
<td>2</td>
<td>276/354</td>
<td>120.5</td>
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<td>VMCP</td>
<td>4</td>
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<td>84.2</td>
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<td>VBMP</td>
<td>3</td>
<td>283/310</td>
<td>136.6</td>
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<td>MOCCA</td>
<td>2</td>
<td>100/185</td>
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<tr>
<td>MCBP</td>
<td>3</td>
<td>374/976</td>
<td>52.2</td>
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<tr>
<td>Other CCT</td>
<td>7</td>
<td>448/630</td>
<td>203.9</td>
<td></td>
</tr>
</tbody>
</table>

Dose Intensity of CCT:

- Lower dose CCT: 15 patients, 121/157, 50.8%
- Higher dose CCT: 12 patients, 130/230, 56.9%

Metyrapone dose and route: Intensive Met: 2 patients, 306/574, 53.1%
- Lower oral Met dose: 14 patients, 1015/1456, 70.1%
- Higher oral Met dose: 11 patients, 1153/1568, 73.3%

Prednisone dose:
- Lower Pred dose: 13 patients, 1069/1531, 69.0%
- Higher Pred dose: 14 patients, 1456/2089, 69.0%

Dose intensity of MP:
- Lower dose MP: 14 patients, 1127/1631, 69.0%
- Higher dose MP: 13 patients, 1398/1970, 70.0%

DISCUSSION

Thirty randomized trials that compared CCT with MP for the treatment of multiple myeloma have been identified. Individual patient data were supplied for 20 of these, which represents approximately 75% of the randomized patients identified. It was possible to extract mortality information from publications for seven of the remaining 10 trials, although these data should be regarded as less reliable, given the limitations that have been demonstrated for the use of such data in other reviews.²

Patients allocated to CCT experience higher response rates than those allocated to MP. The incompleteness of the data available for this end point means that this result should be interpreted with caution. Furthermore, there is no difference in response duration between responders in the CCT and MP groups, and yet the better response rate with CCT does not lead to a survival benefit.

Overall, there is no significant difference in mortality between CCT and MP and the large number of patients...
analyzed makes it likely that if there really is any difference, it will be small and not clinically important. It is possible that this overall null result may conceal differences related to particular types of CCT or MP regimens, or to different types of patient, but investigation of these possibilities within this review did not show any particular subgroups of trials or patients in which the findings were definitely different from the overall result.

Because the MP arm consistently contained only two drugs, it is possible to calculate dose-intensities in a reasonably meaningful way. There was no suggestion that more intensive MP arms were any more, or less, beneficial than less intensive ones, although obviously the comparative dose-intensity of the CCT arm within each trial needs to be taken into account.

Assigning comparative dose-intensities to a heterogeneous set of CCT arms is difficult, but an attempt to do this, if only crudely, produced no evidence of any difference. Given the difficulties of investigating dose-intensity in the CCT arms, it is more informative to group similar CCT regimens together to see if they are more effective than MP. Clearly some types of CCT must be better than others, but identifying them with any certainty is difficult. Three categories of CCT (VMCP + VBAP/VCAP, VMP and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Death/Patients</th>
<th>N</th>
<th>Statistic (O/E)</th>
<th>O.R. &amp; 95%CI</th>
<th>O.R. &amp; 95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
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<td>155/142</td>
<td>-8.0</td>
<td>46.2</td>
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<tr>
<td>100–199</td>
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<td>496/652</td>
<td>-0.9</td>
<td>234.1</td>
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<tr>
<td>&gt;200</td>
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<td>812/1073</td>
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<td>132/1653</td>
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</tbody>
</table>

Fig 4. Mortality in trials of CCT vs MP, by presentation features. Format as in Fig 1.

![Fig 5. Response duration in trials of CCT vs MP. Format as in Fig 2.](image-url)
VBCMP) appeared slightly better than MP, while three categories (other anthracycline regimen, VMCP and MOCCA) appeared worse. However, it is not possible with the available evidence to decide which, if any, of these are real effects and which are simply due to the play of chance.

Two further trials had designs that are relevant when assessing the role of CCT. A trial of ABCM versus melphalan alone showed a benefit for ABCM, while a trial of VMCP versus cyclophosphamide plus prednisone did not demonstrate a significant difference. Since it is not known whether melphalan alone is as effective as MP or whether cyclophosphamide is equivalent to melphalan, these two trials were not included in the analyses reported here. Even if they had been, the mortality difference would have remained nonsignificant (odds reduction, 4%; 95% CI, -10% to +2%).

This overview also demonstrates that, with an appropriate analysis of different patient groups using individual patient data, there is no evidence that CCT is more beneficial to survival than MP in higher risk patients, but less effective in lower risk patients. This is contrary to the previous meta-analysis of published data.

CCT has evolved, especially with respect to high-dose therapy; and it is possible that some of these newer regimens are more effective than MP; but until randomized trials of these combinations are reported, this review provides the most reliable evidence currently available on the relative value of CCT and MP.

REFERENCES

2. Stewart IA, Parmar MKB: Meta-analysis of the literature or individual patient data: Is there a difference? Lancet 341:418-422, 1993
Interferon as therapy for multiple myeloma: an individual patient data overview of 24 randomized trials and 4012 patients

THE MYELOMA TRIALISTS' COLLABORATIVE GROUP Secretariat based at Imperial Cancer Research Fund/Medical Research Council Clinical Trial Service Unit, Radcliffe Infirmary, Oxford, UK

Received 22 December 2000; accepted for publication 16 February 2001

Summary. Many randomized trials have evaluated α-interferon as myeloma therapy, some suggesting a benefit and others not. Most were too small to give reliable answers, so a systematic overview has been performed to provide a more reliable estimate of the effect of interferon. The main end-points were response rates (induction trials), progression-free survival (PFS) and overall survival (OS). Individual patient data were supplied for 24 trials involving 4012 patients, 12 induction trials (2469 patients) and 12 maintenance trials (1543 patients). In induction, response rates were slightly better with interferon (57.5% versus 53.1%, P = 0.01), PFS was better with interferon (33% versus 24% at 3 years, P < 0.00001), an effect seen in both induction (P = 0.0003) and maintenance (P < 0.00001) trials. Median time to progression was increased by about 6 months in both settings. OS was somewhat better with interferon (53% versus 49% at 3 years, P = 0.01) with median survival increased by about 4 months. This benefit was restricted to the smaller trials. The effect of interferon was not significantly related to the dose or duration of interferon or to patients' characteristics. PFS was improved with interferon, but the survival benefit, if any, was small and needs balancing against cost and toxicity.

Keywords: myeloma, interferon, response, progression, survival.

Although patients with multiple myeloma may respond at first to induction chemotherapy, most will eventually suffer progression and death from the disease, irrespective of whether single-agent or combination chemotherapy is used (Myeloma Trialists' Collaborative Group, 1998). In vitro, α-interferon (IFN) has an anti-proliferative effect on myeloma cells (Cooper & Welander, 1987), and there have been many randomized trials of its addition to induction chemotherapy or of its use as maintenance therapy for patients who have already responded to their initial cytotoxic treatment (Bataille & Harousseau, 1997). The majority of these trials have been too small to give reliable answers when taken alone. Most individual trials have not reported a significant improvement in survival, showing that IFN does not have a substantial effect on survival. It might, nevertheless, have a moderate, but still clinically meaningful, benefit (cf. tamoxifen as adjuvant therapy for breast cancer; Early Breast Cancer Trialists' Collaborative Group, 1998). To determine reliably whether or not this is the case, a systematic overview ('meta-analysis') of all available trials is needed. This should preferably involve a central review of the data on each individual patient in each of the trials, not just the review of the published results from those trials (Stewart & Parmar, 1993; Clarke & Godwin, 1998; Fritz & Ludwig, 2000). We report such an overview.

PATIENTS AND METHODS

Standard procedures for overviews based on individual patient data (IPD) were followed (Stewart & Clarke, 1995). Trials in which patients with myeloma were randomized to interferon versus no interferon in either the induction or maintenance phases of therapy were sought in registers and databases [the Cochrane Controlled Trials Register, PDQ (Physician Data Query), MIDLINE and EMBASE]; in abstracts of haematology or oncology meetings; by scrutiny of reports of trials and review articles; and by contact with individual trialists, trial groups and pharmaceutical companies. The cut-off for trial identification was mid 1997.

Baseline data were requested for each randomized patient on date of diagnosis, date of birth (or age), sex, Durie-Salmon stage (Durie & Salmon, 1975), haemoglobin,
### Table I. Presentation features of the individual data patient population.

<table>
<thead>
<tr>
<th>Type of Patient</th>
<th>Induction trials</th>
<th>Maintenance trials</th>
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<tr>
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<td>Number of patients</td>
<td>Percentage* of patients</td>
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<td></td>
<td>IFN</td>
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<td>1239</td>
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<td>101</td>
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<td>50–59</td>
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<td>70+</td>
<td>415</td>
<td>394</td>
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<td>557</td>
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<td>681</td>
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<td>I</td>
<td>76</td>
<td>85</td>
</tr>
<tr>
<td>II</td>
<td>1462</td>
<td>490</td>
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<tr>
<td>III</td>
<td>637</td>
<td>626</td>
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<td>Haemoglobin (g/dl):</td>
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<td>192</td>
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<td>9–10</td>
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<td>138</td>
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<tr>
<td>&gt;12</td>
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<td>191</td>
<td>189</td>
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<td>Platelets (× 10^9/l):</td>
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<td>310</td>
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<td>β2-microglobulin (mg/l):</td>
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<td>Creatinine (mmol/l):</td>
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<td>&gt;129</td>
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<td>223</td>
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<td>IgG</td>
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<td>683</td>
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<tr>
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<tr>
<td>Minimal</td>
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<tr>
<td>Multiple</td>
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<tr>
<td>Complete response</td>
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<tr>
<td>Partial response</td>
<td>—</td>
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<tr>
<td>Stable disease</td>
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</table>

*Percentages may not add up to 100 either because of rounding or because of missing data (for example, performance status is known for 15 + 42 + 11 = 68% of patients in induction trials and is unknown for 32%).


<table>
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<tr>
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<th>Complete plus partial response* (%)</th>
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<td>39</td>
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<tr>
<td>85I EMSG 1985</td>
<td>33</td>
<td>67</td>
<td>93</td>
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<td>86I Rome IFN 1</td>
<td>50</td>
<td>41</td>
<td>95</td>
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<td>86J MGCS 1986</td>
<td>334</td>
<td>22</td>
<td>64</td>
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<tr>
<td>87E EMSG 2 (I)</td>
<td>256</td>
<td>24</td>
<td>56</td>
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<td>87D Royal London</td>
<td>34</td>
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<td>47</td>
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<td>88A ECOG 9486</td>
<td>485</td>
<td>18</td>
<td>66</td>
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<td>89H KIF, Avicenne</td>
<td>282</td>
<td>10</td>
<td>67</td>
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<tr>
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<td>583</td>
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<td>44</td>
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<td>90H ALSG Myeloma II</td>
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<td>41</td>
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<td>72</td>
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<td>41</td>
<td>53</td>
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<tr>
<td>Total</td>
<td>2469</td>
<td>17.1</td>
<td>57.5</td>
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Statistical tests:
- Observed-expected: 17.8
- Variance: 62.7
- Increase in odds of response: 25%
- Effect P-value: 0.02
- Test for heterogeneity between trials: $X^2_o = 16.7, P = 0.08$
- $X^2_1 = 24.2, P = 0.01$

*Response criteria were those used by each group.
References for each trial are listed in the Appendix.

platelets, white blood count, $\beta_2$-microglobulin, M-band type, creatinine, calcium, albumin, bone lesions, performance status, date of randomization and allocated treatment (data were not available for other parameters, e.g. cytogenetics, C-reactive protein, plasma cell labelling index).

The data requested on events after randomization were type (complete or partial) and date of response, progression and its date, most recent status (dead or alive) and date of death or date of last follow-up. The data were checked for obvious inconsistencies and were amended as necessary through correspondence with the responsible trialists. The data used in the analyses were confirmed with the responsible trialists. This manuscript was circulated to all relevant trialists and amended in the light of their comments.

**Statistical methods.** All analyses presented are by allocated treatment (intention to treat). The main statistical methods for combining trials are described in detail elsewhere (Early Breast Cancer Trialists' Collaborative Group, 1990). To summarize, the number of events observed (O) in the interferon group of each trial is compared with the number expected (E) if the events in that trial had been equally distributed between the interferon and no interferon groups. The difference between these numbers, O - E, and its variance yields the log-rank test for each trial. The individual patient data allow these statistics to be calculated using the exact dates of events, which is more statistically reliable and clinically informative than basing the calculations on proportions alive at a particular point in time (Stewart & Parmar, 1993). The sum of the statistics for each trial provides the overall statistics, which are then used to calculate reductions in the odds of death. All P-values are two-tailed. Analyses were first performed in October 1997, with updated analyses over the subsequent few months as data queries were clarified.

The main end-points analysed were: (i) overall survival, (ii) complete response (CR) and complete plus partial response (PR) rates, for induction trials, (iii) progression-free survival, defined either as the time from response to recurrence (in induction trials) or as the time from randomization to recurrence or progression (in maintenance trials) ignoring (i.e. censoring at) death without recurrence or progression, and (iv) survival from progression. The criteria for CR, and PR, were those used in each individual trial. Response duration was also analysed, and was defined in the same way as progression-free survival except that deaths without recurrence were included as events.

**RESULTS**

**Trials and patients**

Thirty-six trials that compared IFN with control were
### Overview of Interferon Therapy for Myeloma

#### Study start year, code and name

<table>
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<tr>
<th>Study start year, code and name</th>
<th>Prog'sions/Patients</th>
<th>IFN</th>
<th>None</th>
<th>Statistics (O-E) Var.</th>
<th>O.R. &amp; CI (IFN : None)</th>
<th>Odds Redn. (SD)</th>
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</thead>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td>85B GATLA 3-M-85</td>
<td>13/16</td>
<td>14/15</td>
<td>-2.6</td>
<td>6.3</td>
<td>34% (33); P = 0.3</td>
<td></td>
</tr>
<tr>
<td>861 Rome IFN 1</td>
<td>16/21</td>
<td>16/19</td>
<td>-4.2</td>
<td>6.5</td>
<td>46% (20); P = 0.1</td>
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</tr>
<tr>
<td>865 MGCS 1986</td>
<td>97/105</td>
<td>61/69</td>
<td>2.9</td>
<td>37.9</td>
<td>-65% (17); P = 0.6</td>
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<tr>
<td>87E EMSG 2 (h)</td>
<td>32/70</td>
<td>37/86</td>
<td>-7.9</td>
<td>16.7</td>
<td>35% (19); P = 0.05</td>
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</tr>
<tr>
<td>88A ECOG 9486</td>
<td>123/161</td>
<td>121/153</td>
<td>-13.9</td>
<td>59.2</td>
<td>21% (12); P = 0.07</td>
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<tr>
<td>89H KIF, Avoncine</td>
<td>43/97</td>
<td>42/95</td>
<td>1.3</td>
<td>21.2</td>
<td>-7% (22); P = 0.8</td>
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<tr>
<td>90D NMSG 04-90</td>
<td>83/125</td>
<td>99/134</td>
<td>-16.4</td>
<td>44.8</td>
<td>31% (13); P = 0.005</td>
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<tr>
<td>90H ALSG Myeloma II</td>
<td>14/24</td>
<td>21/25</td>
<td>-5.0</td>
<td>7.9</td>
<td>64% (22); P = 0.005</td>
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<tr>
<td>90J Ital. NHLSG (I)</td>
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<td>15/21</td>
<td>-4.7</td>
<td>6.0</td>
<td>54% (24); P = 0.06</td>
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<td>91B GMM (I), Mexico</td>
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<td>14/40</td>
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<td>6.9</td>
<td>-11% (49); P = 0.6</td>
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<tr>
<td><strong>Subtotal</strong></td>
<td>451/685</td>
<td>440/638</td>
<td>-52.7</td>
<td>213.4</td>
<td>22% (6) reduction</td>
<td>P = 0.0003</td>
</tr>
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</table>

Test for heterogeneity between trials: $\chi^2_6 = 15.9; P = 0.07$

#### Interferon in maintenance:

<table>
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<th>Study start year, code and name</th>
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<th>Statistics (O-E) Var.</th>
<th>O.R. &amp; CI (IFN : None)</th>
<th>Odds Redn. (SD)</th>
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<tbody>
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<td>85D Ital. MMSG M84</td>
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<td>44/51</td>
<td>-14.0</td>
<td>18.0</td>
<td>54% (16); P = 0.001</td>
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<td>87C SWOG 8824</td>
<td>78/107</td>
<td>83/104</td>
<td>-8.1</td>
<td>39.9</td>
<td>14% (14); P = 0.2</td>
<td></td>
</tr>
<tr>
<td>87D NCI-C MY6</td>
<td>66/89</td>
<td>80/92</td>
<td>-21.1</td>
<td>33.9</td>
<td>46% (12); P = 0.0003</td>
<td></td>
</tr>
<tr>
<td>87H MGWS (extended)</td>
<td>45/61</td>
<td>62/64</td>
<td>-23.5</td>
<td>23.5</td>
<td>63% (13); P = 0.0001</td>
<td></td>
</tr>
<tr>
<td>88B EMSG 2 (m)</td>
<td>22/46</td>
<td>37/54</td>
<td>-8.8</td>
<td>14.6</td>
<td>45% (20); P = 0.02</td>
<td></td>
</tr>
<tr>
<td>88E GMTS MMK2</td>
<td>41/52</td>
<td>58/65</td>
<td>-5.0</td>
<td>24.0</td>
<td>19% (12); P = 0.2</td>
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<tr>
<td>88F Royal Marsonen</td>
<td>31/42</td>
<td>33/42</td>
<td>-8.0</td>
<td>15.5</td>
<td>32% (21); P = 0.01</td>
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<tr>
<td>89A MRC-MYEL-Be</td>
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<td>118/140</td>
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<td>20% (12); P = 0.002</td>
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<tr>
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<td>0.6</td>
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<td>-3% (39); P = 0.6</td>
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<tr>
<td>90B PETHEMA</td>
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<td>-8.7</td>
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<td>38% (16); P = 0.04</td>
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<tr>
<td>90K Ital. NHLSG (M)</td>
<td>30/44</td>
<td>28/48</td>
<td>0.5</td>
<td>14.3</td>
<td>-4% (17); P = 0.5</td>
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<tr>
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<td>36/70</td>
<td>45/66</td>
<td>-10.0</td>
<td>19.4</td>
<td>40% (18); P = 0.02</td>
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<tr>
<td><strong>Subtotal</strong></td>
<td>549/767</td>
<td>635/776</td>
<td>-116.7</td>
<td>289.5</td>
<td>34% (5) reduction</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

Test for heterogeneity between trials: $\chi^2_7 = 21.7; P = 0.03$

Total

<table>
<thead>
<tr>
<th>Study start year, code and name</th>
<th>Prog'sions/Patients</th>
<th>IFN</th>
<th>None</th>
<th>Statistics (O-E) Var.</th>
<th>O.R. &amp; CI (IFN : None)</th>
<th>Odds Redn. (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>1000/1452</td>
<td>1075/1414</td>
<td>-169.4</td>
<td>493.9</td>
<td>29% (4) reduction</td>
<td>P &lt; 0.0001</td>
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</tbody>
</table>

95% CI for total, 90% CI for individual trials

Test for heterogeneity (22 trials): $\chi^2_{22} = 41.0; P = 0.006$

Test for heterogeneity between subtotals: $\chi^2_3 = 3.5; P = 0.06$

Fig 1. Progression in trials of interferon (IFN) versus none. Large squares indicate trials that provide more information and hence have narrower 95% confidence intervals (CI). If the square is to the left of the solid line then progression-free survival is better in the group allocated IFN, but if the CI crosses this line then this result is not statistically significant ($P < 0.01$). An arrow at the right-hand end of the CI indicates that the CI extends further than the plotting area. The subtotals for induction and maintenance trials separately and the overall total are represented as diamonds centred on the odds ratio (OR) estimate, with 95% CI shown by the width of the diamond and with the odds reduction also given as a percentage along with its standard deviation (SD). Vertical dotted lines show the overall odds ratio estimate, i.e. the centre of the diamond. References for each trial are listed in the Appendix.

identified, involving about 4900 patients. Details of these are given in the Appendix. In some of the induction trials, interferon was continued into the maintenance phase (see Appendix). Individual patient data were supplied from 23 trials (4066 patients): 12 in induction (2469 patients) (Prest et al., 1989; Montuoro et al., 1990; Corrado et al., 1991; Atchison et al., 1993; Österborg et al., 1993; Capniet et al., 1994; Ludwig et al., 1995; The Nordic Myeloma Study Group, 1996; Joshua et al., 1997; Oken et al., 1999; GMM(I), Mexico trial, unpublished observations, presented as an
abstract at the IV International Workshop on Multiple Myeloma, 1993; KJR Avicenne trial, unpublished observations; poster 5-1, presented at the VI International Workshop on Multiple Myeloma, Boston, 1997). 12 in maintenance (1543 patients) (Aviles et al. 1991; Capristi et al. 1994; Salmon et al. 1994; Broman et al. 1995; Ludwig et al. 1995; Feest et al. 1995; Westin et al. 1995; Grosbois et al. 1997; Bladé et al. 1998; Cunningham et al. 1998; Drayson et al. 1998; Pulsori et al. 1998) and one in refractory and relapsed disease (54 patients: as these small numbers do not permit reliable analysis, this trial is not considered further) (Gertz et al. 1995). Table I summarizes the presentation features of the 4012 patients in these induction and maintenance trials. There was generally good balance across treatment arms.

Response
Both complete response and complete plus partial response rates were slightly, but significantly, better with IFN (Table II).

Outcome after response
For patients who responded in induction trials, the response duration was significantly better with IFN than without (30% versus 25% at 3 years, log-rank \( P = 0.0005 \)). Similarly, for all patients in maintenance trials, response duration was better with IFN than without (27% versus 19% at 3 years, log-rank \( P < 0.00001 \)). For induction and maintenance trials together, response duration at 3 years was 28% with IFN and 20% without (log-rank \( P < 0.00001 \)). Among those who had not yet suffered disease progression, the death rates (expressed as deaths/person-years) did not differ significantly between the IFN and control groups: 5% versus 6% in induction trials (\( P = 0.7 \)) and 3% versus 4% in maintenance trials (\( P = 0.5 \)). There was, however, a definite effect of IFN on time to progression (censoring at death without progression) (Figs 1 and 2), both in induction trials (\( P = 0.0003 \)) and in maintenance trials (\( P < 0.00001 \)). Median time to progression was increased with IFN by about 6 months in both groups of trials, with large reductions in the risk of progression both in year 1 and in year 2 (odds reductions: 35% (SD 5) and 38% (SD 7), respectively, both log-rank \( P < 0.00001 \)). There was no evidence of any additional benefit beyond year 2, but there was also no evidence that the early benefit was lost (odds increase 2% (SD 12), log-rank \( P = 1.0 \)), based on analysis from the start of year 3.

Survival from progression
Overall, survival from progression was worse for patients who had been allocated IFN than for control patients (22% versus 26% 3 years later, log-rank \( P = 0.02 \)). Although this was significant only in the maintenance trials (odds ratio 1.21, \( P = 0.07 \)) and not in the induction trials (odds ratio 1.04, \( P = 0.6 \)), this apparent difference between these two groups of trials could well be largely or wholly owing to
### Overview of Interferon Therapy for Myeloma

<table>
<thead>
<tr>
<th>Study start year, code and name</th>
<th>Deaths/Patients</th>
<th>IFN</th>
<th>None</th>
<th>O.R. &amp; CI</th>
<th>Odds Redn.</th>
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</thead>
<tbody>
<tr>
<td>Interferon in induction:</td>
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<td>(O-E) Var.</td>
<td></td>
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<tr>
<td>85B GATLA 3–M—85</td>
<td>23/41</td>
<td>26/43</td>
<td>–2.3</td>
<td>13.1</td>
<td>–16% (95); P = 0.5</td>
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<tr>
<td>85I EMSG 1985</td>
<td>4/15</td>
<td>5/18</td>
<td>–0.7</td>
<td>2.2</td>
<td>–29% (95); P = 0.6</td>
</tr>
<tr>
<td>85F Rome IFN 1</td>
<td>18/22</td>
<td>26/28</td>
<td>–9.9</td>
<td>9.4</td>
<td>–62% (95); P = 0.001</td>
</tr>
<tr>
<td>85J MGCS 1986</td>
<td>146/164</td>
<td>149/170</td>
<td>–0.6</td>
<td>73.3</td>
<td>–1% (95); P = 0.9</td>
</tr>
<tr>
<td>87C EMSG 2 (I)</td>
<td>59/125</td>
<td>67/131</td>
<td>–6.1</td>
<td>31.1</td>
<td>–10% (95); P = 0.3</td>
</tr>
<tr>
<td>87D Royal London</td>
<td>6/17</td>
<td>10/17</td>
<td>–2.0</td>
<td>5.9</td>
<td>–41% (95); P = 0.3</td>
</tr>
<tr>
<td>95A ECOG 9486</td>
<td>19/1245</td>
<td>192/240</td>
<td>0.4</td>
<td>86.1</td>
<td>–0% (95); P = 1</td>
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<tr>
<td>95H KIF, Avicenne</td>
<td>75/145</td>
<td>71/137</td>
<td>2.5</td>
<td>36.2</td>
<td>–7% (95); P = 0.7</td>
</tr>
<tr>
<td>95Q NMSG 04–90</td>
<td>218/286</td>
<td>233/297</td>
<td>–6.1</td>
<td>112.6</td>
<td>–5% (95); P = 0.6</td>
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<tr>
<td>95H ALSG Myeloma II</td>
<td>32/59</td>
<td>33/54</td>
<td>–4.7</td>
<td>16.5</td>
<td>–26% (95); P = 0.5</td>
</tr>
<tr>
<td>95J Ital. NHLSG (I)</td>
<td>9/56</td>
<td>13/51</td>
<td>–3.2</td>
<td>5.4</td>
<td>–45% (95); P = 0.2</td>
</tr>
<tr>
<td>95B GMM (I), Mexico</td>
<td>30/75</td>
<td>29/73</td>
<td>–0.7</td>
<td>13.2</td>
<td>–5% (95); P = 0.8</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td><strong>815/1320</strong></td>
<td><strong>854/1239</strong></td>
<td><strong>–33.5</strong></td>
<td><strong>410.9</strong></td>
<td><strong>8% (95) reduction P = 0.1; NS</strong></td>
</tr>
</tbody>
</table>

Test for heterogeneity between trials: $X^2_{11} = 14.6; P = 0.2; NS$

### Interferon in maintenance:

<table>
<thead>
<tr>
<th>Study start year, code and name</th>
<th>Deaths/Patients</th>
<th>IFN</th>
<th>None</th>
<th>O.R. &amp; CI</th>
<th>Odds Redn.</th>
</tr>
</thead>
<tbody>
<tr>
<td>85D Ital. MMSG M84</td>
<td>40/50</td>
<td>43/51</td>
<td>–4.6</td>
<td>20.5</td>
<td>–20% (95); P = 0.3</td>
</tr>
<tr>
<td>87C SWOG 8624</td>
<td>89/107</td>
<td>77/104</td>
<td>6.4</td>
<td>41.3</td>
<td>–17% (95); P = 0.3</td>
</tr>
<tr>
<td>87D NCI-C MY6</td>
<td>66/89</td>
<td>75/92</td>
<td>–10.8</td>
<td>34.5</td>
<td>–27% (95); P = 0.07</td>
</tr>
<tr>
<td>87H MGVMS (extended)</td>
<td>39/61</td>
<td>41/64</td>
<td>–1.4</td>
<td>19.6</td>
<td>–7% (95); P = 0.4</td>
</tr>
<tr>
<td>88B EMSG 2 (M)</td>
<td>14/45</td>
<td>25/54</td>
<td>–4.3</td>
<td>9.4</td>
<td>–57% (95); P = 0.2</td>
</tr>
<tr>
<td>88C GMGT GMM02</td>
<td>42/52</td>
<td>50/65</td>
<td>6.5</td>
<td>21.6</td>
<td>–36% (95); P = 0.2</td>
</tr>
<tr>
<td>88P Royal Macond</td>
<td>17/42</td>
<td>21/42</td>
<td>–4.8</td>
<td>9.1</td>
<td>41% (95); P = 0.5</td>
</tr>
<tr>
<td>89A MFPC—MYEL—6e</td>
<td>103/143</td>
<td>99/140</td>
<td>1.7</td>
<td>49.0</td>
<td>–3% (95); P = 0.8</td>
</tr>
<tr>
<td>89C CMN (M), Mexico</td>
<td>5/13</td>
<td>4/8</td>
<td>–0.2</td>
<td>0.8</td>
<td>–22% (95); P = 0.8</td>
</tr>
<tr>
<td>90B PETHENA</td>
<td>27/60</td>
<td>28/62</td>
<td>–5.2</td>
<td>13.3</td>
<td>–52% (95); P = 0.2</td>
</tr>
<tr>
<td>95K Ital. NHLSG (M)</td>
<td>19/44</td>
<td>15/48</td>
<td>–2.2</td>
<td>8.7</td>
<td>–23% (95); P = 0.4</td>
</tr>
<tr>
<td>95E GERM</td>
<td>25/70</td>
<td>37/76</td>
<td>–10.2</td>
<td>15.1</td>
<td>–48% (95); P = 0.005</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td><strong>489/767</strong></td>
<td><strong>519/776</strong></td>
<td><strong>–32.5</strong></td>
<td><strong>243.5</strong></td>
<td><strong>12% (95) reduction P = 0.04</strong></td>
</tr>
</tbody>
</table>

Test for heterogeneity between trials: $X^2_{11} = 17.2; P = 0.1; NS$

### Total

<table>
<thead>
<tr>
<th>Deaths/Patients</th>
<th>IFN</th>
<th>None</th>
<th>O.R. &amp; CI</th>
<th>Odds Redn.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1295/1997</strong></td>
<td><strong>1373/2015</strong></td>
<td><strong>–56.0</strong></td>
<td><strong>654.4</strong></td>
<td><strong>10% (4) reduction</strong></td>
</tr>
</tbody>
</table>

95% CI for total, 99% CI for individual trials

Test for heterogeneity (24 trials): $X^2_{23} = 32.2; P = 0.1; NS$
Test for heterogeneity between subtotals: $X^2_{1} = 0.4; P = 0.5; NS$

Fig 3. Mortality in trials of interferon (IFN) versus none. Format as Fig 1.

---

chance, as there was no significant heterogeneity between the two results ($P = 0.2$).

**Overall survival**

Survival was significantly [odds reduction 10% (SD 4), log-rank $P = 0.01$] improved with IFN (Figs 3 and 4). Although there was not a statistically significant benefit in the induction trials [odds reduction 8% (SD 5), log-rank $P = 0.1$], while there was in the maintenance trials [odds reduction 12% (SD 6), log-rank $P = 0.04$], there was again no significant heterogeneity between these two results ($P = 0.5$). Increases in median survival of about 2 and 7 months were observed in the induction and maintenance trials, respectively, with overall an increase of about 4 months.

**Subgroup analyses**

Subgroup analyses were performed to explore whether the benefit of IFN was greater for some patients than for others, and whether IFN was superior in particular therapeutic situations.

Types of patient
The relative benefit of IFN on time to progression for the 12 presentation parameters listed in Table I is shown in Fig. 5. There was no evidence, from any of these variables, that the benefit of IFN was importantly greater or worse in either good- or poor-risk patients. When the induction and maintenance trials were analysed separately, there was similarly no evidence of important differences between types of patient in their response to IFN within either category of trials. Nor was there evidence of heterogeneity between patient groups with respect to survival, either overall or within induction and maintenance trials separately (data not shown). In the maintenance trials, there was no evidence that the benefit of IFN differed between patients who were in CR, in PR or who had stable disease at the time of randomization (Fig 6).

Type of therapy
The trials can be grouped by certain features of their designs: for example, dose of IFN, duration of IFN, and type of concurrent or prior chemotherapy. This yielded no significant heterogeneity with respect to progression (Fig 7), or with respect to response rate (in the induction trials) or overall survival. There was one feature of the trials that produced a substantial trend, namely that the magnitude of the benefit of IFN on mortality was much greater in small trials than in larger ones (Fig 8), with no clear benefit for IFN in the latter. Similarly, there was a trend for the effects of IFN on progression-free survival to be better in the smaller trials, although there was still a significant benefit in the larger trials (Fig 8).

Trials for which individual patient data were not supplied
It was possible to extract data on response rates from reports of a further five trials (Cooper et al., 1993; Aviles et al., 1995; Rome IFN2 and Spanish Coop Group trials, unpublished observations, presented as abstracts, p299 and p301, at the 24th Congress of the International Society of Haematology, London, 1992; Buenos Aires trial, unpublished observations, presented as an abstract, p321, at the 18th International Congress of Chemotherapy, Stockholm, 1993). For two of which an estimate of the survival benefit could also be made. Inclusion of these data did not materially alter the results, with the benefit of interferon on both response rates and overall survival becoming slightly greater.

DISCUSSION
Overall effect of interferon
This overview of 24 randomized trials, using individual patient data, does demonstrate some clinical benefit for interferon as a treatment for myeloma. Response rates were slightly better when interferon was used during induction treatment (although the definition of response varied between studies, this analysis is valid as patients allocated interferon are only compared with controls in the same
Overview of Interferon Therapy for Myeloma 1027

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prog'rsions/Patients</th>
<th>Statistics</th>
<th>O.R. &amp; CI*</th>
<th>Odds Redn.</th>
</tr>
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<td>(O-E) Var.</td>
<td>(IFN : None)</td>
<td>(SD)</td>
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</tr>
<tr>
<td>&lt;60</td>
<td>319/448</td>
<td>322/430</td>
<td>-29-4</td>
<td>142-3</td>
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<td>60-69</td>
<td>295/556</td>
<td>441/588</td>
<td>-81-0</td>
<td>191-5</td>
</tr>
<tr>
<td>70+</td>
<td>289/418</td>
<td>308/395</td>
<td>-50-9</td>
<td>134-6</td>
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<tr>
<td>Test for trend within subgroup: X^2_1 = 2.1; P = 0.1; NS</td>
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<td></td>
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<tr>
<td>Sex:</td>
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</tr>
<tr>
<td>Female</td>
<td>443/676</td>
<td>469/627</td>
<td>-75-1</td>
<td>214-9</td>
</tr>
<tr>
<td>Male</td>
<td>545/773</td>
<td>603/786</td>
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<td>265-9</td>
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<td>Test for heterogeneity within subgroup: X^2_1 = 0.0; P = 0.9; NS</td>
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<td>-18-6</td>
<td>28-5</td>
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<tr>
<td>Stage II</td>
<td>283/432</td>
<td>308/413</td>
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<tr>
<td>Stage III</td>
<td>509/716</td>
<td>544/716</td>
<td>-75-2</td>
<td>245-4</td>
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<td>Hboglobin:</td>
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<td>12.0+</td>
<td>264/390</td>
<td>299/394</td>
<td>-50-3</td>
<td>129-5</td>
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<tr>
<td>11.0-11.9</td>
<td>150/220</td>
<td>160/202</td>
<td>-28-2</td>
<td>69-5</td>
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<tr>
<td>9.0-10.9</td>
<td>257/357</td>
<td>281/331</td>
<td>-33-5</td>
<td>118-4</td>
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<tr>
<td>&lt;9.0</td>
<td>118/167</td>
<td>141/184</td>
<td>-22-8</td>
<td>55-5</td>
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<td>Test for trend within subgroup: X^2_1 = 0.1; P = 0.7; NS</td>
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<tr>
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<td>346/479</td>
<td>372/482</td>
<td>-56-5</td>
<td>163-2</td>
</tr>
<tr>
<td>5.0-7.4</td>
<td>251/365</td>
<td>282/363</td>
<td>-40-3</td>
<td>124-0</td>
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<td>7.5+</td>
<td>159/221</td>
<td>180/228</td>
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<td>71-1</td>
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<td>144-1</td>
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<td>321/472</td>
<td>380/486</td>
<td>-59-8</td>
<td>161-1</td>
</tr>
<tr>
<td>&lt;150</td>
<td>118/161</td>
<td>127/184</td>
<td>-20-1</td>
<td>51-2</td>
</tr>
<tr>
<td>Test for trend within subgroup: X^2_1 = 0.0; P = 0.9; NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

95% CI for total and subtotals, 99% CI for individual groups

Fig 5. Progression in trials of interferon (IFN) versus none by patient presentation features. Format as Fig 1.


trial). Also, in both induction and maintenance trials, progression of the disease was delayed. Once progression did occur, subsequent survival was slightly worse in those previously given interferon, so the effect on progression was greater than the effect on survival. Overall therefore, there was at most only a small survival benefit.

There is a suggestion that the progression-free survival curve for the interferon arm might be showing a plateau consistently above that in the control arm (Fig 2), but the number of patients at risk beyond 3 years is still relatively small and longer follow-up will be required to determine reliably whether this really is the case. There is currently no apparent plateau in the survival curve (Fig 4).

The results presented here, based on intention-to-treat analyses of all randomized patients, will underestimate the true potential benefit of interferon as there will have been a
The Myeloma Trialists' Collaborative Group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prognostic/Patients</th>
<th>Statistics</th>
<th>O.R. &amp; CI*</th>
<th>Odds Redn.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN</td>
<td>None</td>
<td>(O-E) Var.</td>
<td>(IFN : None)</td>
</tr>
<tr>
<td>b2-microglobulin:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4.0</td>
<td>339/490</td>
<td>355/488</td>
<td>-37.0</td>
<td>165.6</td>
</tr>
<tr>
<td>4.0-7.9</td>
<td>106/264</td>
<td>206/208</td>
<td>-25.3</td>
<td>91.8</td>
</tr>
<tr>
<td>8.0+</td>
<td>92/130</td>
<td>118/141</td>
<td>-26.2</td>
<td>43.9</td>
</tr>
<tr>
<td>Test for trend within subgroup: $X^2 = 3.9; P = 0.05$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100</td>
<td>407/580</td>
<td>448/556</td>
<td>-86.2</td>
<td>198.5</td>
</tr>
<tr>
<td>100-129</td>
<td>178/249</td>
<td>184/229</td>
<td>-22.8</td>
<td>81.9</td>
</tr>
<tr>
<td>130+</td>
<td>171/239</td>
<td>214/271</td>
<td>-32.3</td>
<td>81.4</td>
</tr>
<tr>
<td>Test for trend within subgroup: $X^2 = 0.3; P = 0.6; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.5</td>
<td>431/625</td>
<td>476/600</td>
<td>-31.0</td>
<td>215.5</td>
</tr>
<tr>
<td>2.5-2.9</td>
<td>226/303</td>
<td>244/313</td>
<td>-35.1</td>
<td>106.4</td>
</tr>
<tr>
<td>&gt;3.0</td>
<td>75/104</td>
<td>83/99</td>
<td>-9.4</td>
<td>33.5</td>
</tr>
<tr>
<td>Test for trend within subgroup: $X^2 = 0.3; P = 0.6; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M band type:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>273/386</td>
<td>294/365</td>
<td>-39.6</td>
<td>130.9</td>
</tr>
<tr>
<td>IgG</td>
<td>545/782</td>
<td>578/767</td>
<td>-34.0</td>
<td>265.0</td>
</tr>
<tr>
<td>Light chain</td>
<td>97/157</td>
<td>104/147</td>
<td>-26.4</td>
<td>42.7</td>
</tr>
<tr>
<td>Other</td>
<td>26/44</td>
<td>34/46</td>
<td>-7.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Test for heterogeneity within subgroup: $X^2 = 4.6; P = 0.2; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone lesions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>143/229</td>
<td>173/333</td>
<td>-32.8</td>
<td>70.4</td>
</tr>
<tr>
<td>Minimal</td>
<td>191/282</td>
<td>220/287</td>
<td>-38.4</td>
<td>94.2</td>
</tr>
<tr>
<td>Multiple</td>
<td>412/561</td>
<td>426/547</td>
<td>-67.7</td>
<td>192.8</td>
</tr>
<tr>
<td>Test for trend within subgroup: $X^2 = 0.7; P = 0.4; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Performance status:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>146/238</td>
<td>180/249</td>
<td>-27.9</td>
<td>76.0</td>
</tr>
<tr>
<td>Minimal symptoms</td>
<td>271/445</td>
<td>354/465</td>
<td>-56.3</td>
<td>155.5</td>
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<tr>
<td>Symptomatic</td>
<td>78/102</td>
<td>65/81</td>
<td>-7.0</td>
<td>26.3</td>
</tr>
<tr>
<td>Test for trend within subgroup: $X^2 = 0.0; P = 0.6; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>995/1452</td>
<td>1072/1414</td>
<td>-170.7</td>
<td>492.0</td>
</tr>
</tbody>
</table>

"95% CI for total and subtotals, 99% CI for individual groups"

Test 2P < 0.00001

Fig 5. Continued.

degree of non-compliance with allocated treatment. However, this has the advantage that it represents the situation in regular clinical practice in which, inevitably, some patients will stop taking a drug that has side-effects.

A recent meta-analysis based on published data (Pritz & Ludwig, 2000) showed similar results to those reported here, with estimates of a 6.6% better response rate with interferon and median improvements of 4-6 months in relapse-free and 3-7 months in overall survival respectively (cf. 4-6%, 6 months and 4 months, respectively, in the present report). IPD overviews are much more time-consuming and labour-intensive than published data meta-analyses, but have a number of advantages. These include being able to assess trial quality, to include all randomized patients, to use updated data with longer follow-up, to investigate effects over time, to allow more

### Overview of Interferon Therapy for Myeloma

#### Disease status

<table>
<thead>
<tr>
<th>Disease status</th>
<th>Events/Patients</th>
<th>Statistics (O−E) Var.</th>
<th>O.R. &amp; C.I. (IFN : None)</th>
<th>Odds Redn. (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progression:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>259/360</td>
<td>279/344</td>
<td>-53-0 126-5</td>
<td></td>
</tr>
<tr>
<td>Partial response</td>
<td>135/185</td>
<td>154/178</td>
<td>-30-1 66-3</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>58/76</td>
<td>68/89</td>
<td>-5-8 29-5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>549/767</td>
<td>635/776</td>
<td>-117-7 280-5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(71.8%)</td>
<td>(81.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for trend within subgroup: $\chi^2_1 = 0.6; P = 0.4; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Mortality:

<table>
<thead>
<tr>
<th>Disease status</th>
<th>Events/Patients</th>
<th>Statistics (O−E) Var.</th>
<th>O.R. &amp; C.I. (IFN : None)</th>
<th>Odds Redn. (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete response</td>
<td>227/360</td>
<td>229/344</td>
<td>-20-1 109-7</td>
<td></td>
</tr>
<tr>
<td>Partial response</td>
<td>117/185</td>
<td>117/178</td>
<td>3-6 54-7</td>
<td></td>
</tr>
<tr>
<td>Stable disease</td>
<td>42/76</td>
<td>59/89</td>
<td>-9-3 24-2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>480/767</td>
<td>519/776</td>
<td>-32-5 243-5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(62.6%)</td>
<td>(58.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test for trend within subgroup: $\chi^2_1 = 0.0; P = 0.8; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig 6. Outcome in maintenance trials by disease status at entry. Format as Fig 1.

### Dose of IFN:

<table>
<thead>
<tr>
<th>Dose of IFN</th>
<th>Events/Patients</th>
<th>Statistics (O−E) Var.</th>
<th>O.R. &amp; C.I. (IFN : None)</th>
<th>Odds Redn. (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;12 MU/week</td>
<td>435/683</td>
<td>470/679</td>
<td>-65-1 217-9</td>
<td></td>
</tr>
<tr>
<td>12+ MU/week</td>
<td>527/719</td>
<td>565/693</td>
<td>-57-7 257-6</td>
<td></td>
</tr>
<tr>
<td>Test for heterogeneity within subgroup: $\chi^2_1 = 0.6; P = 0.4; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Duration of IFN:

<table>
<thead>
<tr>
<th>Duration of IFN</th>
<th>Events/Patients</th>
<th>Statistics (O−E) Var.</th>
<th>O.R. &amp; C.I. (IFN : None)</th>
<th>Odds Redn. (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1 year</td>
<td>169/318</td>
<td>193/316</td>
<td>-27-6 86-7</td>
<td></td>
</tr>
<tr>
<td>1+ years</td>
<td>218/327</td>
<td>240/315</td>
<td>-38-8 107-2</td>
<td></td>
</tr>
<tr>
<td>To progression</td>
<td>555/717</td>
<td>688/701</td>
<td>-95-0 272-7</td>
<td></td>
</tr>
<tr>
<td>Test for heterogeneity within subgroup: $\chi^2_1 = 0.1; P = 0.9; NS$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Total           | 1000/1452        | 1075/1414              | -169-4 493-9             |                |
|                 | (68.5%)          | (78.6%)               |                          |                |

Fig 7. Progression in trials of interferon (IFN) versus none by scheduled dose and duration of interferon. Format as Fig 1. The cut-off points between higher and lower dose categories were selected so that about half the trials were in each group.

Fig 8. Effect of trial size on effect of interferon (IFN). Format as Fig 1. Trial size was defined by the variance obtained in the mortality analysis (with the number of deaths in parentheses): very small = 0–9% (30–39); small = 10–19% (40–50); medium = 20–39% (50–69); large = 40–49% (70–99).

Flexibility of patient subgroup analyses, to permit a consensus paper approved by all relevant trialists and, in this case, to use more standard meta-analysis methods.

A tendency for the smaller trials to indicate a greater survival benefit has also been seen in clinical trials in other diseases (CLASP Collaborative Group, 1994; Fourth International Study of Infarct Survival Collaborative Group (ISIS-4), 1995). The reasons are unclear, but one possible explanation is that there is a publication bias in which unreported results from small trials never got reported, while promising ones do (Egger et al., 1997). We have vigorously sought to identify, and obtain data from, unpublished studies and so this seems unlikely to be a substantial source of bias. Another potential source of bias in smaller trials is less rigorous application of appropriately strict randomization procedures, but as protection against this we have obtained and extensively checked individual patient data from each trial without finding any indication of problems. Inclusion of published data from the five induction trials without IPD did not change the results or their interpretation in any important fashion. Two of the three maintenance trials with no data available were more recent studies that were still open, so this reason for exclusion is unlikely to introduce a bias. If there is any publication bias, or if the smaller studies are for some reason less reliable, then the true survival benefit for interferon would be even less than that observed, and there might actually be little or no benefit. This effect of trial size is the only factor identified that might explain some of the heterogeneity observed between trials (Fig 1), as there are no clear differences in the effect of interferon between patient types and trial designs (see below).

Effect of interferon in different types of patient
Although the overall benefit of interferon is relatively modest, there might be recognizable subgroups of patients who gain larger or smaller benefits from interferon. Twelve prognostic factors have been investigated, but there was no evidence that the benefit of interferon differed substantially between types of patients. In particular, there was no evidence that good-risk patients obtained a greater benefit than poorer-risk ones, or that patients in complete response at the time of maintenance randomization benefited more than those in partial response or with stable disease. With multiple comparisons being performed, it is probable that the effect of IFN will appear larger or smaller in some subgroups simply owing to the play of chance. It cannot therefore be concluded that patients with high β2-microglobulin or light
## Appendix. Details of trials of interferon as therapy for myeloma.

<table>
<thead>
<tr>
<th>Year code</th>
<th>Trial name</th>
<th>Reference</th>
<th>Entry period</th>
<th>Scheduled IFN dose</th>
<th>Scheduled IFN duration</th>
<th>Prior/concurrent chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induction trials with individual patient data supplied</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85I</td>
<td>GATLA 3-M-85</td>
<td>Corrado et al., 1991</td>
<td>1985-89</td>
<td>5 MU/m² sec. d 1,3,5,8,10,12/28 d</td>
<td>1 year</td>
<td>MP</td>
</tr>
<tr>
<td>85I</td>
<td>EMSG 1985</td>
<td>Preti et al., 1989</td>
<td>1985-86</td>
<td>2 MU × 3/w</td>
<td>6-12 months</td>
<td>VMCP</td>
</tr>
<tr>
<td>86I</td>
<td>Rome IFN 1</td>
<td>Montecucco et al., 1990</td>
<td>1986-87</td>
<td>5 MU d 1,3,5,8,10,12/28 d</td>
<td>2 years or progression</td>
<td>MP</td>
</tr>
<tr>
<td>86I</td>
<td>MCGS 1986</td>
<td>Osterborg et al., 1993</td>
<td>1986-91</td>
<td>7 MU/m² d 1-5/21 d to response then 3 MU/m² 3 d/w to progression</td>
<td>To progression</td>
<td>MP</td>
</tr>
<tr>
<td>87E</td>
<td>EMSG2 (I)</td>
<td>Ludwig et al., 1995</td>
<td>1987-93</td>
<td>2 MU × 3/w</td>
<td>To CR + 3 cycles, PR + 6 cycles, or stable disease + 9 cycles</td>
<td>VMCP</td>
</tr>
<tr>
<td>87O</td>
<td>Royal London</td>
<td>Alston et al., 1993</td>
<td>1987-90</td>
<td>3 MU × 3/w w 1.2/3 w</td>
<td>If no response by 3 months</td>
<td>C</td>
</tr>
<tr>
<td>88A</td>
<td>ECG 9486</td>
<td>Oben et al., 1999</td>
<td>1988-91</td>
<td>5 MU/m² × 3/w (w 1-3/6 w)</td>
<td>2 years</td>
<td>VMCP/VPAP</td>
</tr>
<tr>
<td>89H</td>
<td>KIE Aviscense</td>
<td>Poster presentation, VIII International Workshop on Multiple Myeloma, 1997</td>
<td>1989-91</td>
<td>3 MU/m² × 3/w in 2 w between 3 w courses</td>
<td>To plateau</td>
<td>VMCP</td>
</tr>
<tr>
<td>90D</td>
<td>NMSS 04-90</td>
<td>The Nordic Myeloma Study Group, 1996</td>
<td>1990-93</td>
<td>5 MU × 3/w</td>
<td>To failure</td>
<td>MP</td>
</tr>
<tr>
<td>90H</td>
<td>ALSG Myeloma II</td>
<td>Joshua et al., 1997</td>
<td>1990-92</td>
<td>3 MU d from 3rd month</td>
<td>To progression</td>
<td>CBAP</td>
</tr>
<tr>
<td>90J</td>
<td>Italian NHLSO (I)</td>
<td>Capristi et al., 1994</td>
<td>1990-92</td>
<td>3 MU × 3/w</td>
<td>9 months</td>
<td>MP</td>
</tr>
<tr>
<td>91B</td>
<td>GMM (I), Mexico</td>
<td>Abstract presentation, IV International Workshop on Multiple Myeloma, 1993</td>
<td>1991-97</td>
<td>5 MU d 1,3,5,7,9,11,13,15,17</td>
<td>To progression</td>
<td>MP or VMCP</td>
</tr>
</tbody>
</table>

### Induction trials with no individual patient data received, but published data available

<table>
<thead>
<tr>
<th>Year code</th>
<th>Trial name</th>
<th>Reference</th>
<th>Entry period</th>
<th>Scheduled IFN dose</th>
<th>Scheduled IFN duration</th>
<th>Prior/concurrent chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>85A</td>
<td>CALGB 8415</td>
<td>Cooper et al., 1993</td>
<td>1985-89</td>
<td>2 MU/m² d 1,3,5,8,10,12/28 d</td>
<td>2 years</td>
<td>MP</td>
</tr>
<tr>
<td>87L</td>
<td>CMN (I), Mexico</td>
<td>Ariles et al., 1995</td>
<td>1987-90</td>
<td>5 MU × 3/w</td>
<td>1 year</td>
<td>VMCP, CVN, BEV</td>
</tr>
<tr>
<td>89G</td>
<td>Rome IFN 2</td>
<td>Abstract presentation, 24th Congress of the International Society of Haematology, 1992</td>
<td>1989-91</td>
<td>6 MU d 1,3,5,8,10,12</td>
<td>?</td>
<td>MP</td>
</tr>
<tr>
<td>90G</td>
<td>Spanish Coop Grp</td>
<td>Abstract presentation, 24th Congress of the International Society of Haematology, 1992</td>
<td>1997</td>
<td>7 MU/m² d 4/21 to response then 3 MU/m² 3 d/7 d</td>
<td>To progression</td>
<td>MP</td>
</tr>
</tbody>
</table>

### Maintenance trials with individual patient data supplied

<table>
<thead>
<tr>
<th>Year code</th>
<th>Trial name</th>
<th>Reference</th>
<th>Entry period</th>
<th>Scheduled IFN dose</th>
<th>Scheduled IFN duration</th>
<th>Prior/concurrent chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>85D</td>
<td>Italian MMSG M84</td>
<td>Pulsoni et al., 1994</td>
<td>1985-88</td>
<td>3 MU/m² × 3/w</td>
<td>To progression</td>
<td>VMCP and VPAP (ind)</td>
</tr>
<tr>
<td>85C</td>
<td>SWOG 8624</td>
<td>Salmon et al., 1994</td>
<td>1986-90</td>
<td>3 MU × 3/w</td>
<td>To progression</td>
<td>VAD, VMCP and VPAP (ind)</td>
</tr>
<tr>
<td>87D</td>
<td>NCIC-C MY6</td>
<td>Brown et al., 1995</td>
<td>1987-92</td>
<td>2 MU/m² (starting dose) × 3/w</td>
<td>To progression</td>
<td>MP (ind)</td>
</tr>
<tr>
<td>87H</td>
<td>MOWS (extended)</td>
<td>Wexin et al., 1995</td>
<td>1987-90</td>
<td>5 MU × 3/w</td>
<td>To progression</td>
<td>MP (ind)</td>
</tr>
<tr>
<td>88B</td>
<td>EMSG 2 (M)</td>
<td>Ludwig et al., 1993</td>
<td>1988-91</td>
<td>2 MU × 3/w</td>
<td>To progression</td>
<td>VMCP + IFN (ind)</td>
</tr>
<tr>
<td>88E</td>
<td>GMG M9002</td>
<td>Peet et al., 1995</td>
<td>1988-91</td>
<td>5 MU d 1,3,5,8,10,12</td>
<td>To progression</td>
<td>MP then VEMAD (ind)</td>
</tr>
<tr>
<td>88F</td>
<td>Royal Marsden</td>
<td>Cunningham et al., 1998</td>
<td>1988-91</td>
<td>3 MU/m² × 3/w</td>
<td>To progression</td>
<td>CVAP then HD M and ABMT (ind)</td>
</tr>
<tr>
<td>89A</td>
<td>MRC-MYEL-66</td>
<td>Bravinson et al., 1996</td>
<td>1989-93</td>
<td>3-6 MU × 3/w</td>
<td>To progression</td>
<td>ABCM, MP, VAD, VMCP/VPAP, or HD M (ind)</td>
</tr>
<tr>
<td>89E</td>
<td>CMN (M), Mexico</td>
<td>Ariles et al., 1991</td>
<td>1989-90</td>
<td>5 MU × 3/w</td>
<td>18 months</td>
<td>? (ind), MP or VMCP (maint)</td>
</tr>
<tr>
<td>90B</td>
<td>PETHEMA</td>
<td>Bladé et al., 1998</td>
<td>1990-94</td>
<td>3 MU/m² × 3/w</td>
<td>To progression</td>
<td>VMCP and VPAP (ind)</td>
</tr>
<tr>
<td>90K</td>
<td>Ital. NHLSO (M)</td>
<td>Capristi et al., 1994</td>
<td>1990-94</td>
<td>3 MU × 2/w</td>
<td>To progression</td>
<td>MP + IFN (ind)</td>
</tr>
<tr>
<td>91E</td>
<td>GERM</td>
<td>Gresbovits et al., 1997</td>
<td>1991-95</td>
<td>3 MU/m² × 3/w</td>
<td>2 years</td>
<td>MP or VMCP (ind)</td>
</tr>
</tbody>
</table>

*Overview of Interferon Therapy for Myeloma, 1013*
chain disease (both relatively small subgroups, with wide confidence intervals that overlap the overall effect estimate) obtain greater benefit from IFN.

**Effect of trial design**

The trials included in the overview differed substantially in their design and there was evidence of heterogeneity between trials with respect to the effect of interferon. It was not, however, possible to reliably identify any aspect of study design that materially affected the size of the benefit of interferon. The use of interferon in induction led to a prolongation of response duration among patients who achieved a response (either partial or complete), as did its use in maintenance among all patients entered. Although the survival benefit for induction trials was not statistically significant (P = 0.1), while that for maintenance trials was of borderline significance (P = 0.04), this does not provide good evidence that the survival benefit is restricted to the maintenance setting, as there was no significant heterogeneity of benefit between the induction trials and the maintenance trials. There was no evidence that the scheduled dose or duration of interferon was important.

Only one maintenance trial used interferon after high-dose therapy (Cunningham et al., 1998). The point estimate result for this one trial (32% odds reduction for progression) was consistent with the overall effect in the maintenance setting (34% odds reduction). This trial was, however, small with wide confidence intervals (95% CI: 74% odds reduction to 10% odds increase), so a greater, or indeed lesser, benefit for interferon in the context of high-dose therapy compared with other forms of chemotherapy cannot be ruled out. More randomized evidence is needed if this issue is to be addressed reliably and the results of a large ongoing intergroup trial in the USA are awaited.

**Other factors**

Interferon is an expensive drug and can have substantial side-effects that adversely affect a patient’s quality of life. One study estimated a cost of S110 000 per quality-adjusted life-year gained (Nord et al., 1997). Information on side-effects was not collected for this overview, but the relatively large Nordic trial of interferon during induction did include a quality-of-life assessment (Wisloff et al., 1996), which showed an adverse impact of interferon during the first year of treatment. Against this must be set the fact that interferon keeps patients progression-free for longer and therefore free of the symptoms of myeloma, which may (at least for a time) improve the patients’ quality of life, although the Nordic quality-of-life study did not find a late benefit. A survey of myeloma patients (Ludwig et al., 1997) showed that approximately half the patients interviewed would be willing to tolerate the side-effects of interferon if it improved progression-free or overall survival by 6 months.

**CONCLUSION**

This overview shows that interferon delays disease progression and that, even after several years, there still
appears to be a persistent reduction in the likelihood of having suffered recurrence. If this does eventually translate into a real difference in mortality, then it is possible that a few more years of follow-up of these same trials and an updated overview of them may yield a much more definite mortality advantage than is indicated by the current survival analyses. Until such evidence becomes available, the current analysis shows only a small survival benefit, if any, and decisions as to whether to use interferon or not will need to balance a modest clinical benefit on progression and mortality against cost and toxicity.

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The Myeloma Trialists’ Collaborative Group


Lancet. 351, 1451–1467.


KEYNOTE ADDRESS

The role of vertebral augmentation in multiple myeloma: International Myeloma Working Group Consensus Statement


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Introduction

There are approximately 20,000 new patients diagnosed with myeloma in the United States each year.1 With the availability of better treatments and resultant improved survival, there are currently close to 100,000 patients living with myeloma in the United States. Similar incidence and prevalence rates exist throughout Europe.2 Of these patients, the spine is affected by osteolytic and/or osteoblastic bone disease in 70%.3 Myeloma is the commonest primary cancer affecting the spine. Painful vertebral compression fractures (VCFs) affect approximately 30% of myeloma patients. As myeloma patients live longer, it is especially relevant to provide the best available treatment for pain and reduce disabilities that can result from VCFs.4

The focus of this summary is to assess the role of minimally invasive percutaneous injection of polymethyl methacrylate (PMMA), first developed as ‘vertebroplasty’ in France in the late 1980s. Considerable experience accrued, especially in Europe, with the use of vertebroplasty as treatment for painful VCFs. The fractured bone fragments are stabilized and strengthened by PMMA and pain is substantially improved. A more recent modification of vertebroplasty is percutaneous balloon kyphoplasty whereby inflation of a balloon prior to PMMA injection can restore vertebral height and reduce kyphotic deformity in addition to stabilizing the fractured vertebral body.

The first prospective trial evaluating the role of balloon kyphoplasty in multiple myeloma showed that over 80% of the treated patients experienced significant pain control.5 In addition, there was an overall 30% height restoration with improvement of 65-70% of height restoration when the procedure was performed for fractures less than 6 months old. The procedure was also noted to be effective and safe in other malignancies.6 In another study of 20 multiple myeloma patients (48 levels) treated with balloon kyphoplasty, significant pain improvement as judged by visual analog scale occurred within the first year of follow-up.7 About 80% of patients with initial kyphotic deformity had post-operative kyphosis correction of approximately 6°, with only minimal loss of height after 1 year (~1.8°). The overall data related to both vertebroplasty and balloon kyphoplasty are addressed in a number of publications.5,8-10

In considering the potential benefit of PMMA injection, it is necessary to be aware of the biomechanics of pathologic spine fractures (Figure 1). With the occurrence of a VCF, the center of gravity moves forward. Because of the large bending moment created, the anterior spine, especially in the regions adjacent to the VCF, must resist larger compressive stresses. The posterior muscles and ligaments are additionally stressed, which can be an obvious source of pain. Early intervention is a way to reduce the risk of a ‘domino effect’ with increased forward movement of the center of gravity, additional compressive stresses and possible further VCFs. The consequences of progressively altered vertebral mechanics and the kyphosis-related VCFs in myeloma patients can be substantial as summarized in Table 1.

Obviously, the safety of vertebral augmentation is an important consideration. The potential complications of vertebral augmentation are summarized in Table 2. A literature review meta-analysis of procedure-related complications for balloon kyphoplasty and vertebroplasty indicates that complications such as extravasation of PMMA are less with balloon kyphoplasty.21 It should be noted that asymptomatic extravasation of

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15See Appendix for members of the International Myeloma Working Group
Received 18 February 2008; revised 2 April 2008; accepted 28 April 2008
The role of vertebral augmentation in multiple myeloma
MA Husain et al

Figure 1 Movement of center of gravity (CG) forward with vertebral compression fracture.

Table 1 Consequences of VCF-related kyphosis

<table>
<thead>
<tr>
<th>Consequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compression of abdominal contents</td>
<td>Anorexia, weight loss</td>
</tr>
<tr>
<td>Decreased lung capacity</td>
<td>Limited exercise tolerance/physical activity</td>
</tr>
<tr>
<td>Anterior loading of spine (Figure 1)</td>
<td>Subsequent fractures</td>
</tr>
<tr>
<td></td>
<td>Increasing kyphosis and deformity</td>
</tr>
</tbody>
</table>

Abbreviation: VCF, vertebral compression fracture.

Table 2 Potential complications of vertebral augmentation

<table>
<thead>
<tr>
<th>Complication</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extravasation of PMMA cement</td>
<td>Local effects</td>
</tr>
<tr>
<td></td>
<td>Systemic effects including pulmonary</td>
</tr>
<tr>
<td>Card compression (spinal cord)</td>
<td></td>
</tr>
<tr>
<td>Pneumothorax</td>
<td></td>
</tr>
<tr>
<td>Retroperitoneal hematoma</td>
<td></td>
</tr>
<tr>
<td>Infection: local/systemic</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: PMMA, polymethyl methacrylate.

*aSee text for discussion.*

Table 3 Outcomes with balloon kyphoplasty at H Lee Moffitt Cancer Center*

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>41 patients: 62 kyphoplasties</td>
<td></td>
</tr>
<tr>
<td>13% PMMA extravasation</td>
<td></td>
</tr>
<tr>
<td>1 case of pneumothorax resolved</td>
<td></td>
</tr>
<tr>
<td>95% partial or substantial pain relief</td>
<td></td>
</tr>
<tr>
<td>All patients discharged within 23 h (i.e., &lt; 1 day)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: PMMA, polymethyl methacrylate.

*aData published under Vrioni et al.*

PMMA occurs in about 7% balloon kyphoplasty versus 19.7% vertebroplasty but rarely leads to clinical complications.\(^{22}\) As an example, the single center experience at Moffitt Cancer Center gives a realistic expectation as to outcomes in a center specializing in myeloma care (Table 1). The presence of any plasmacytoma tissue between the PMMA and the fractured cortical bone can lead to suboptimal improvement in stabilization and any subsequent pain relief from any form of vertebral augmentation.\(^{23}\) Active awareness of potential complications and careful patient selection are obviously crucial.

The role of vertebral augmentation in the treatment of myeloma of the spine is still evolving. The impact of VCFs upon quality of life and survival is illustrated by results of a large study in women aged ≥65 years. In this study of a total of 9575 women aged 65 years or older, 1915 of the women (20.0%) were diagnosed as having VCF secondary to osteoporosis. The fractures were not only associated with increased morbidity, but also with increased mortality.\(^{23}\) The increased mortality was particularly from pulmonary complications.\(^{23}\) Moreover, patient mortality increased with greater numbers of vertebral fractures, from 19 per 1000 woman-years in women with no fractures to 44 per 1000 woman-years in those with five or more fractures (P<0.001).

These data accentuate the need for management guidelines for VCFs. Formal guidelines on the use of vertebral augmentation for myeloma in the spine are missing. The purpose of this paper is to review the evidence regarding the role of vertebral augmentation in the spine and to provide a consensus statement on the role of vertebral augmentation for the management of myeloma affecting the spine. Those aspects of therapy were reviewed, discussed, and considered by the International Myeloma Working Group and a special advisory board convened at the time of the XI International Myeloma Workshop KOS Greece on 29 June 2007.

The following is the consensus statement from the International Myeloma Working Group:

1. **Indications for vertebral augmentation:** The indications are summarized in Table 4. These indications apply, provided contraindications are not present as summarized in Table 5. If severe pain is present, the advisory board reached a consensus that it is very reasonable to proceed with immediate vertebral augmentation. A major advantage is the rapid pain relief especially compared with alternative analgesic strategies summarized in Table 6. Early augmentation also proactively reduces the risk of the vicious cycle of further VCFs as described above as well as providing the maximum chance of restoring height and correcting angular deformity. Early augmentation also does not preclude additional or subsequent use of any of the alternative strategies such as those summarized in Table 6. In the absence of severe pain, augmentation is a proactive measure to preserve structural integrity. When there is severe bone destruction and in anticipation of potential long patient survival, this secondary indication is very much a viable option to maximize quality of life by preventing potential VCFs.

2. **Identification of patients suitable for vertebral augmentation:** The four major components of the recommended baseline evaluation are summarized in Table 7. Obviously, one must be certain that the pain is emanating from the collapsed or damaged vertebrae(s). In addition to X-ray, which most patients will have undergone to diagnose the problem, more detailed evaluation with magnetic resonance imaging (including STIR (Short T1 Inversion Recovery) images) is essential particularly to determine the presence or absence of spinal cord compression and/or edema. Also, the potential for retropulsion and/or direct leakage of PMMA can be
Table 4  Indications for vertebral augmentation

Primary: severe pain present (pain > 7/10 on VAS)
- Collapse of one or more vertebrae (VCF)
- Bone destruction (osteolytic/osteosclerotic) with high risk of collapse of one or more vertebrae

Secondary: severe pain absent (pain < 7/10 on VAS)
- Significant loss of height and/or structural integrity or stability

Abbreviations: VAS, visual analogue scale; VCF, vertebral compression fractures.
*Provided no contraindications (Table 5).

Table 5  Contraindications to vertebral augmentation

<table>
<thead>
<tr>
<th>Absolute</th>
<th>Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraindications to general or local anesthesia</td>
<td>Lesions above T3</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Osteoblastic metastases</td>
</tr>
<tr>
<td>Bleeding disorder</td>
<td>Patient &lt;40 years of age</td>
</tr>
<tr>
<td>Infection at the site</td>
<td>Technically not feasible</td>
</tr>
<tr>
<td>Pain unrelated to vertebral collapse</td>
<td>(vertebra plana)</td>
</tr>
<tr>
<td>Card compression</td>
<td>Fractures with obstructing plasmaoma(s)</td>
</tr>
<tr>
<td>Presence of overt instability</td>
<td>Retropulsed bone</td>
</tr>
<tr>
<td>Severe cardiopulmonary insufficiency</td>
<td></td>
</tr>
<tr>
<td>Allergy to procedure-related drugs/contrast</td>
<td></td>
</tr>
</tbody>
</table>

Table 6  Alternatives for pain therapy

<table>
<thead>
<tr>
<th>Options</th>
<th>Discussion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA vertebral augmentation</td>
<td>Rapid pain relief</td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>Simple procedure</td>
</tr>
<tr>
<td>Systemic anti-myeloma therapy</td>
<td>Pain relief has slower onset and less complete</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>Reduces tumor mass swelling and may eliminate plasmaoma(s) locally</td>
</tr>
<tr>
<td>Analgesics</td>
<td>Can be used acting and effective</td>
</tr>
</tbody>
</table>

Table 7  Identification of patients suitable for vertebral augmentation

2. MRI is essential to document the anatomy and assess spinal cord edema/compression.
3. Assessment of myeloma disease status and potential anti-myeloma treatment needs.
4. Assessment of other pain therapy options (Table 6).

Abbreviation: MRI, magnetic resonance imaging.

3. Timing for the vertebral augmentation: Early intervention is currently being investigated in the CAFE (Cancer Fracture Evaluation) trial in which immediate and delayed vertebral augmentation are being contrasted and compared. The group reviewed available data and clinical experience available now and agreed to the following:

- Immediate vertebral augmentation is a treatment option for acute VCF with severe pain or VCF at high risk for progressive deformity. Excellent short- and long-term results have been achieved in this setting.
- For patients with lesser pain and/or vertebral damage, a trial of analgesic therapy with supportive measures including bisphosphonates and systemic therapy is generally recommended. The appropriate duration of this type of therapeutic trial relates to the severity of the pain and potential reversibility with systemic measures. In general, augmentation can be considered as safe and feasible especially if pain worsens and/or persists and/or to prevent further vertebral collapse. Early intervention is especially important if stabilizing the spinal structure and/or restoring the height are critical. Excellent results have been achieved in these settings. Results of the ongoing randomized CAFE trial evaluating pain relief and quality of life with immediate versus delayed balloon kyphoplasty are eagerly awaited.
- If pain persists at the site of VCF, there is no upper time limit beyond which augmentation cannot be considered. However, earlier intervention is preferred to achieve maximal stabilization and/or correction of deformities.

4. Number of levels to be considered for treatment:

- Multiple augmentation procedures may be necessary and appropriate. In general, three to four vertebrae per intervention is considered reasonable and feasible during a single procedure (if required). As many as 16 augmentations have been performed on an individual myeloma patient in separate sessions or stages.
- Vertebral augmentation for adjacent or suspect vertebrae without fracture may be necessary. Such augmentations can be considered when there is a fracture with kyphosis in the thoracolumbar region because the stress due to the deformity in this region is very high. It is particularly common to consider the performance of an additional augmentation procedure in a vertebra when it is located between two fractured vertebrae such as T11 and L1 requiring treatment for T12 to avoid post-procedure T12 collapse.

5. The highest level of augmentation to be performed was briefly discussed. It was decided that experienced operators can perform vertebral augmentation to levels as high as the cervical area and that this can be effective and safe. For practical purposes, T5-L5 is the range that can be performed safely by the percutaneous route.
6. The method of vertebral augmentation: The risk of complications, especially the risk of PMMA leakage, is greater with vertebroplasty. However, it was agreed that both the utility and the likelihood of clinically significant complications are very much dependent upon the experience of the operator. It is therefore recommended that the choice of balloon kyphoplasty versus vertebroplasty be left to the discretion of the operator and be based upon the goals of the procedure.

7. Use of vertebral augmentation versus radiation therapy: Vertebral augmentation is considered the procedure of choice to improve quality of life for painful VCFs. However, external beam radiotherapy (EBR) is a valid option that requires careful consideration. EBR is simple and can be performed in one session without risks of anesthesia, bleeding, infections or compromise of vital structures. Local marrow stem cell damage is most likely minimal different with 30 Gy of EBR versus the impact of heated PMMA injected into one or three vertebral bodies. Thus, if discreet plasma talk massa exist within a vertebra and/or there is a symptomatic extramedullary mass or impending/over spinal cord compression occurs, the use of EBR can be the option of choice. Systemic antimyeloma therapy is an alternative for rapid reduction in myeloma tumor burden. In addition, medical pain therapy can provide helpful relief as necessary.

8. Physical rehabilitation: To maximize the recovery from augmentation, a physical rehabilitation program is recommended. Ideally, this should be in the form of water aerobics and thoracicolumbar stabilization with an extension directed focus, under the supervision of a physical therapist.

9. Further trials are still required to clarify the role of vertebral augmentation in a variety of situations including the following:

- prevention of further fractures in asymptomatic patients;
- treating asymptomatic fractures; and
- treating asymptomatic fractures compromising the spinal structure or pulmonary capacity

Discussion

This International Myeloma Working Group Consensus Statement is the latest in a series of publications from the working group as summarized in Table 8. Additional consensus statements scheduled for 2008 include the role of free light chain analysis; guidelines for use of Epoetin; the role of imaging; and a new genetic classification for myeloma.

Vertebral augmentation techniques discussed as part of this consensus statement can be used for both acute and chronic fractures. Myeloma patients with mechanical pain, that is pain that is most significant in the upright position, standing or walking and significantly decreased in a reclining position, which anatomically correlates with the area of the fracture, are most likely to benefit from vertebral augmentation. Other types or causes of pain (radicular, dyesthetic, discogenic or degenerative) should be carefully assessed as they can coexist with pain related to compression fractures and are unlikely to respond to vertebral augmentation interventions.

Absolute contraindications to augmentation including myelopathy or cauda equina syndrome are listed in Table 5. Relative contraindications include coagulopathy, neutropenia, allergy to substances used for the procedure and high anesthetic risks (Table 5).

Table 8: International Myeloma Working Group Consensus Statements

<table>
<thead>
<tr>
<th>Article</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Criteria for classification of monoclonal gammopathies</td>
<td>Br J Haematol 2003; 121:748-57</td>
</tr>
<tr>
<td>Myeloma Management Guidelines</td>
<td>Hematology Journal 2003;4579-96</td>
</tr>
<tr>
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<td>JCO 2006; 32(13):1-9</td>
</tr>
<tr>
<td>International Uniform Response</td>
<td>Leukemia 2008 (1-7)</td>
</tr>
<tr>
<td>Criteria for Multiple Myeloma</td>
<td>Mayo Clinic Proceedings 2007; 88:516-22</td>
</tr>
<tr>
<td>Use of Bisphosphonates in Myeloma</td>
<td>Leukemia 2008; 22:244-23</td>
</tr>
<tr>
<td>Prevention of Thrombosis and Leukemia</td>
<td>Blood, April 2006; 111(9): 4039-47</td>
</tr>
</tbody>
</table>

Currently, there are no specific guidelines or contraindications regarding factors such as kyphosis, retropulsion and caudal compromise or degree of vertebral body collapse. The issue of spinal instability and its effect on overall decision making need to be determined on an individual basis by the treating spine expert. The unstable spine is at risk of progressive deformity or impairment of the neural elements. Generally, operative care with open surgery rather than percutaneous augmentation techniques is most valuable in the setting of spinal instability. In addition, the number of fractures that can be treated at each intervention or stage; the amount of methylmethacrylate to be injected; the need to treat adjacent or intervening nonfractured segments; the issue of unilateral or bilateral, transpedicular or extra-pedicular; open or percutaneous approaches, under local or general anesthesia all need to be determined by the treating surgeon or radiologist. However, it is recommended that not more than three or four fractures are treated per stage as the risk of pulmonary complications increases with the number of treated fractures. The advisory board delegated the details of this decision making, related to numbers and methodology, to the selected operators of the augmentation procedures.

In general, vertebral augmentation is not recommended for asymptomatic fractures unless there is documented increased collapse and deformity progression or in the context of an adjacent or intervening segment. This could be documented by follow-up radiologic studies performed every 3 months or sooner if the clinical picture dictates. The role of skeletal augmentation for sacral or iliac fractures in patients with myeloma is currently unknown.

As noted above, vertebral augmentation should be considered as the procedure of choice to improve quality of life for painful compression spinal fractures in myeloma patients instead of EBR or placement of intrathecal morphine pumps. In patients with plasmacytoma in bone or extramedullary plasmacytoma extending into the epidural space, open surgical decompression or radiation therapy with or without augmentation may be appropriate. In patients with a stable spine without fracture or progressive deformity, radiotherapy should be considered first. Receiving radiation therapy does not preclude future augmentation. Vertebral augmentation and radiation can be viewed as complementary, with augmentation restoring anatomy and radiation ablationing symptomatic disease. Augmentation has the advantage of rapid relief in a single sitting, which should allow patients requiring radiation to be treated in a more comfortable state. This is particularly important for individuals undergoing
radiosurgery where treatment time approaches an hour and immobility is crucial for accuracy.

In summary, patients with plasmacytoma, extramedullary masses and cord compromise should be considered for use of up-front radiotherapy. Vertebral augmentation is a developing field with current and future trials being necessary to further define what constitutes an 'impending fracture' and establish the role of pre-emptive augmentation procedures. Having all options available for multiple myelomas, patients insures optimal therapeutic intervention to improve both quality of life and overall survival.

References


Appendix

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Leukemia
Mobilization in myeloma revisited: IMWG consensus perspectives on stem cell collection following initial therapy with thalidomide-, lenalidomide-, or bortezomib-containing regimens

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The past decade has witnessed a paradigm shift in the initial treatment of multiple myeloma with the introduction of novel agents such as thalidomide, lenalidomide, and bortezomib, leading to improved outcomes. High-dose therapy and autologous stem cell transplantation remains an important therapeutic option for patients with multiple myeloma eligible for the procedure. Before the advent of the novel agents, patients underwent stem cell collection prior to significant alkylating agent exposure, given its potential deleterious effect on stem cell collection. With increasing use of the novel agents in the upfront setting, several reports have emerged raising concerns about their impact on the ability to collect stem cells. An expert panel of the International Myeloma Working Group (IMWG) was convened to examine the implications of these therapies on stem collection in patients with myeloma and to develop recommendations for addressing these issues. Here we summarize the currently available data and present our perspective on the problem and potential options to overcome this problem. Specifically, we recommend early mobilization of stem cells, preferably within the first 4 cycles of initial therapy, in patients treated with novel agents and encourage participation in clinical trials evaluating novel approaches to stem cell mobilization. (Blood. 2009;114:1729-1735)

High-dose therapy and autologous stem cell transplantation for multiple myeloma

High-dose therapy and autologous stem cell transplantation (ASCT) remains an integral component of the myeloma treatment algorithm for patients considered eligible for the procedure. The majority of the randomized clinical trials have demonstrated a superior progression-free survival among patients undergoing ASCT compared with those treated with conventional therapies and ASCT was associated with superior overall survival in 3 of those.1-7 Subsequent randomized trials have further defined the role of ASCT by demonstrating equivalent overall survival for delayed transplantation compared with upfront ASCT, albeit with some compromise in the quality-of-life parameters.8 Introduction of novel agents such as thalidomide, lenalidomide, and bortezomib have resulted in a paradigm change in the therapy of myeloma.8-14 The high response rates with these agents, hitherto seen only in the context of high-dose therapy, have once again raised questions regarding the utility of ASCT in the setting of myeloma. Given the lack of long-term follow-up of patients treated with these new agents, the durability of these responses as well as their potential long-term adverse effects remain unknown and ASCT continues to be an important part of myeloma therapy.

Despite the increased use of the newer drugs for the initial treatment of myeloma, there is a continuous increase in the number of ASCTs reported to the Center for International Blood and Marrow Transplant Research (CIBMTR), highlighting their continued important role. Currently, the novel agents appear best suited to be used as first-line therapy, enhancing the quality of responses prior to proceeding to ASCT and diminishing early mortality from

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the disease, or in selected patients as primary therapy, moving ASCT to a second-line position or as adjuncts to transplant conditioning regimens or as maintenance therapy in patients undergoing ASCT. Furthermore, in a randomized trial evaluating single versus double ASCT a survival advantage with tandem ASCT was demonstrated in an unplanned subset analysis for those patients not obtaining at least a very good partial response after the first ASCT. This observation has increased the number of ASCTs being performed for patients not achieving very good partial response after the first ASCT. In addition, ASCT can also be used as part of second-line therapy after relapse, especially among patients who achieved a durable response after the first ASCT.

The traditional approach to patients with newly diagnosed myeloma, considered to be candidates for ASCT, has been to provide initial therapy with 4 to 6 cycles of a non-alkylator-containing regimen followed by collection of stem cells and high-dose therapy. The initial therapy for the disease allows time to obtain necessary insurance approvals as well as control disease-related symptoms, simultaneously controlling toxicity by limiting the number of cycles. In addition, adequate disease control provides an opportunity to reverse disease-related complications where feasible, and generally improve the functional status of the patient, allowing for a safer transplantation. Until the advent of the novel agents, the initial therapy regimens commonly used were vincristine, doxorubicin, and dexamethasone (VAD) or single-agent dexamethasone, both of which shared the advantage of having little impact stem cell mobilization and collection. Previous studies had shown that alkylation agents can potentially affect the stem cell pool and thus interfere with the ability to collect adequate numbers of stem cells.

The number of CD34+ cells collected for ASCT is dependent on a number of factors, most importantly the number of intended transplantations. The cell collection target usually depends on patient age, upfront versus delayed ASCT, patient preference, patient performance status, and presence of comorbidities, among others. Traditionally, the target for CD34 cell collection for a single ASCT has been 4 to 6 x 10^6 CD34+ cells, with studies showing a deleterious impact on engraftment characteristics once the number falls below 2 x 10^6 CD34+ cells. Use of more CD34 cells has not been consistently associated with any significant benefit in the parameters studied. Patient age is an important factor from several perspectives. Clearly there is decreasing use of stem cell transplantation (SCT) with increasing patient age, although selected patients with good performance status may undergo transplantation into their mid-70s. The target for stem cell collection is usually based on single transplantation in the United States, because Medicare reimburses only single SCT for myeloma. Finally, there is a clear impact of age on the ability to collect stem cells, with decreasing yield with advancing age. In the majority of patients undergoing an ASCT for myeloma, stem cells are collected from the peripheral blood following mobilization using growth factor administration with or without preceding chemotherapy. A minority of patients undergo ASCT with stem cells collected through a bone marrow harvest. Use of cyclophosphamide or other chemotherapy regimens for myelosuppression to achieve rebound CD34+ cell spillover into the blood with enhanced effects of myeloid growth factors during the recovery phase of peripheral blood counts allows for a more rapid stem cell collection and higher numbers of collected CD34+ cells compared with myeloid growth factor alone. Institutions differ in their standard approach for collecting stem cells, with pros and cons to either approach. Use of cyclophosphamide, although allowing better stem cell collection and less likelihood of a collection failure (less than the 2 x 10^6 CD34+ cells), prolongs the collection process while awaiting count recovery and increases the risk of febrile neutropenia and other infectious complications.

One of the recent advances in the field of stem cell mobilization strategies has been the introduction of AMD3100 (plerixafor), a receptor chemokine receptor 4 (CXCR4) inhibitor. Previous studies have highlighted the role of CXCR4 in the stem cell mobilization induced by granulocyte colony-stimulating factor (G-CSF) and cyclophosphamide. Lisovski et al showed that mobilization of stem cells by G-CSF coincides in vivo with the cleavage of the N-terminal of the chemokine receptor CXCR4 on the stem cells in the BM, leading to loss of chemotaxis in response to the CXCR4 ligand, the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). In addition, accumulation of serum proteases led to cleavage and inactivation of SDF-1. Originally developed as an anti-HIV drug, the ability of this drug to enhance peripheral mobilization of CD34+ cells was subsequently recognized. AMD3100, a reversible inhibitor of the binding of stromal cell-derived factor-1α (SDF-1α, also known as CXCL12) to its cognate CXCR4, has been shown to increase the number of circulating CD34+ cells in healthy volunteers when administered alone or with G-CSF prior to treatment. Stem cells express CXCR4 and are known to migrate to the bone marrow through a chemokine ligand of SDF-1α that is produced locally by bone marrow stromal cells. Once in the marrow, it is also believed that stem cell CXCR4 can act to help "anchor" these cells to stromal cell surface SDF-1α. AMD3100-induced leukocytosis and elevations in circulating hematopoietic progenitor cell levels are thought to result from a disruption of the CXCR4/CXCL12 interaction and cell adhesion effects, resulting in the appearance of both mature and pluripotent cells in the systemic circulation. AMD3100 has been shown to exert an additive effect on the number of circulating hematopoietic stem and progenitor cells when administered with G-CSF. AMD3100-3102 was a multicenter randomized, double-blind, placebo-controlled comparative trial designed to examine the ability of 240 μg/kg AMD3100 plus G-CSF versus placebo plus G-CSF to mobilize CD34+ stem cells in patients with multiple myeloma (MM), who had not previously failed stem cell collections and had not received previous stem cell transplant. The primary end point, the percentage of patients who achieved 6 x 10^6 or more CD34+ cells/kg in 2 or fewer apheresis days, was met in 106 (72%) of 128 patients in the AMD plus G-CSF group and 53 (34%) of 154 patients in the placebo plus G-CSF group, (P < .001). Fifty-four percent of study patients reached target after 1 day of apheresis in the AMD + G-CSF group compared with 17.3% in the placebo plus G-CSF group. After 4 days of apheresis, these numbers were 86.8% and 56%, respectively.

**Thalidomide-, Lenalidomide-, or Bortezomib-based Regimens as Initial Therapy for Multiple Myeloma**

Introduction of thalidomide represented the first major therapeutic advance in myeloma in several decades. After the initial trials in relapsed myeloma, several randomized phase 3 trials of thalidomide and dexamethasone in patients with previously untreated myeloma were performed. Thalidomide combinations were associated with superior response rates and improved response.
### Table 1. Studies of novel agents with data available for success of stem cell collection

<table>
<thead>
<tr>
<th>Reference</th>
<th>Treatment regimen</th>
<th>No.</th>
<th>Clinical trial</th>
<th>Mobilization regimen</th>
<th>CD34 yield, x10^6/kg</th>
<th>P</th>
<th>Days of leukopheresis</th>
<th>Failed collection % (definition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breilke et al.</td>
<td>TAD</td>
<td>93</td>
<td>Phase 3</td>
<td>CAD</td>
<td>6.8 (2.33-6.0)</td>
<td>.02</td>
<td>1 (1-6)</td>
<td>4 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>105</td>
<td></td>
<td></td>
<td>10.9 (3.8-10)</td>
<td>.001</td>
<td>1 (1-6)</td>
<td>1 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(GSMG-HD3)</td>
<td></td>
<td></td>
<td></td>
<td>1 (1-6)</td>
<td>7 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Breilke et al.</td>
<td>TAD</td>
<td>100</td>
<td>Phase 3</td>
<td>CAD</td>
<td>7.4 (0.9-8.0)</td>
<td>.001</td>
<td>1 (1-6)</td>
<td>3 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>100</td>
<td></td>
<td></td>
<td>9.6 (6.0-8.0)</td>
<td>.001</td>
<td>1 (1-6)</td>
<td>5 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Reisman et al.</td>
<td>TAD</td>
<td>96</td>
<td>Phase 3</td>
<td>VEA00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10 (NA)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>100</td>
<td></td>
<td></td>
<td>9.4 (6.0-8.0)</td>
<td>.001</td>
<td>1 (1-6)</td>
<td>5 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Cavo et al.</td>
<td>TAD</td>
<td>100</td>
<td>Matched pair</td>
<td>CTX + G-CSF</td>
<td>7.85</td>
<td>.01</td>
<td>2</td>
<td>17 (&lt; 4 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>100</td>
<td>analysis</td>
<td></td>
<td>10.5</td>
<td></td>
<td>2</td>
<td>12 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Kumar et al.</td>
<td>TAD</td>
<td>76</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>9.6 (1-18)</td>
<td>.001</td>
<td>3 (1-6)</td>
<td>1 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>22</td>
<td></td>
<td></td>
<td>9.8 (2.1-18.0)</td>
<td>.001</td>
<td>4 (1-10)</td>
<td>1 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>TAD</td>
<td>90</td>
<td></td>
<td></td>
<td>10.0 (5.5-30.1)</td>
<td>.001</td>
<td>4 (1-10)</td>
<td>0 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>40</td>
<td></td>
<td></td>
<td>7.9 (0.5-15.0)</td>
<td>.001</td>
<td>5 (1-12)</td>
<td>3 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Mazumder et al.</td>
<td>TAD</td>
<td>28</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>5.4 (0-17.5)</td>
<td>.001</td>
<td>43 (2 &lt; 10^6/kg)</td>
<td>43 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>41</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>7.4</td>
<td></td>
<td>43 (2 &lt; 10^6/kg)</td>
<td>43 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Rajkumar et al.</td>
<td>TAD</td>
<td>233</td>
<td>Phase 3</td>
<td>VEA00</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>3 (NA)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>220</td>
<td></td>
<td></td>
<td>5.1</td>
<td></td>
<td>3 (NA)</td>
<td>3 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Kumar et al.</td>
<td>TAD</td>
<td>92</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>7.9 (5-16)</td>
<td>.001</td>
<td>11 (5 &lt; 10^6/kg)</td>
<td>11 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Mark et al.</td>
<td>TAD</td>
<td>11</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>8.6 (2-21)</td>
<td>.001</td>
<td>4 (1-10)</td>
<td>3 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>TAD</td>
<td>19</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>3.1 (2.2-4.6)</td>
<td>.001</td>
<td>4 (1-10)</td>
<td>0 (&lt; 2.5 x 10^6/kg)</td>
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<tr>
<td></td>
<td>VAD</td>
<td>137</td>
<td>Retrospective</td>
<td>CTX + G-CSF</td>
<td>14.2 (4-8-255)</td>
<td>.001</td>
<td>3 (1-6)</td>
<td>9 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>157</td>
<td>Retrospective</td>
<td>(4 with G-CSF + AMG3010)</td>
<td>7.3 (4-7-2.5)</td>
<td>.001</td>
<td>3 (1-6)</td>
<td>9 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Popat et al.</td>
<td>TAD</td>
<td>64</td>
<td>Retrospective</td>
<td>G-CSF</td>
<td>7.3 (4-7-2.5)</td>
<td>.001</td>
<td>2 (1-11)</td>
<td>1.5 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>238</td>
<td></td>
<td></td>
<td>26 (2 &lt; 10^6/kg in 4 days)</td>
<td>NA</td>
<td>26 (&lt; 2.5 x 10^6/kg in 4 days)</td>
<td>4 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Jagannath et al.</td>
<td>TAD</td>
<td>8</td>
<td></td>
<td></td>
<td>15.2 (7.2-16.1)</td>
<td>.001</td>
<td>3 (2-5)</td>
<td>0 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Harousseau et al.</td>
<td>TAD</td>
<td>240</td>
<td>Phase 3</td>
<td>G-CSF</td>
<td>6.8</td>
<td>.001</td>
<td>2.0 (mean)</td>
<td>4 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>242</td>
<td></td>
<td></td>
<td>6.4</td>
<td>.001</td>
<td>2.0 (mean)</td>
<td>4 (&lt; 2.5 x 10^6/kg)</td>
</tr>
<tr>
<td>Sonneveld et al.</td>
<td>TAD</td>
<td>150</td>
<td>HOVON-05</td>
<td>CTX + G-CSF</td>
<td>10.4 (4-37)</td>
<td>.001</td>
<td>1 (1-4)</td>
<td>1 (1-4)</td>
</tr>
<tr>
<td></td>
<td>VAD</td>
<td>150</td>
<td></td>
<td></td>
<td>9.06 (4.1-37.6)</td>
<td>.001</td>
<td>1 (1-4)</td>
<td>1 (1-4)</td>
</tr>
<tr>
<td>Richardson et al.</td>
<td>TAD</td>
<td>22</td>
<td>Phase 2</td>
<td>GCSF</td>
<td>4.2</td>
<td>.001</td>
<td>8.7</td>
<td>8.7 (&lt; 2.5 x 10^6/kg)</td>
</tr>
</tbody>
</table>

**TAD indicates thalidomide, doxorubicin, and dexamethasone; VAD, vincristine, doxorubicin, and dexamethasone; CAD, cyclophosphamide 1 gm² per day; x, on day 1; doxorubicin 15 mg² per day, x, on days 1 to 4; dexamethasone 40 mg os, days 1-4, and granulocyte colony-stimulating factor (G-CSF); NA, not available; TAD, thalidomide, dexamethasone; CTX, cyclophosphamide; LD, lenalidomide, dexamethasone; LO, lenalidomide and weekly dexamethasone; BD, bortezomib and dexamethasone.**

*Information on whether a stem cell harvest was attempted was available only for 70% of patients, among whom 37% attempted stem cell harvest. Collection details were not available.

A detailed description of stem cell collection regarding cell counts and definition of failure were not available and are likely to represent a mix of practices given the multicenter nature of the trial.

duration with no definite impact on the overall survival compared with dexamethasone alone or VAD. This was followed by introduction of the thalidomide analog, lenalidomide, that in phase 2 trials resulted in very high response rates as well as deeper responses than seen with previous approaches. Subsequent phase 3 trials of lenalidomide and dexamethasone demonstrated its superiority compared with dexamethasone alone as well as its ability to spare high doses of steroids and simultaneously improve survival. More recent clinical trials have examined the efficacy and tolerability of lenalidomide combined with cyclophosphamide (CTX), bortezomib, and clarithromycin, as well as other combinations. Another major advance in the field had been the introduction of the proteasome inhibitor bortezomib, which along with dexamethasone or in combination with conventional chemotherapy agents is increasingly being used in the setting of newly diagnosed disease with high efficacy. Phase 3 trials of bortezomib in combination with dexamethasone with or without doxorubicin has shown excellent tolerability and improved response rates and progression-free survival compared with traditional VAD in the setting of initial therapy prior to SCT. Both lenalidomide and bortezomib have been combined with cyclophosphamide in the setting of transplantation-eligible patients in phase 2 studies with excellent response rates. Recently reported phase 2 trials have examined the efficacy of combining bortezomib with lenalidomide or thalidomide with or without cyclophosphamide in the setting of newly diagnosed MM. These combinations have led to very high complete and very good partial response rates and will undoubtedly become integral components of the initial treatment choices in the future. This in turn has led to renewed interest in the potential impact of initial therapy on the ability to collect adequate numbers of stem cells for one or more transplantsations.

### Impact of thalidomide, lenalidomide, and bortezomib on peripheral blood stem cell collection

A large volume of data, albeit limited to single institution reports and less detailed data from phase 3 trials, has appeared in the past few years evaluating the effect of these drugs on the stem cell collection process (Table 1). Although there are contradictory data on the impact of thalidomide on stem cell mobilization and collection, the effect if any appears to be relatively small with...
limited impact on the ability to proceed with SCT. In addition, there is no evidence to suggest that initial therapy with thalidomide-containing regimens prior to stem cell collection adversely impacts the engraftment potential of the collected stem cells.

In contrast to thalidomide, one of the common adverse effects of lenalidomide has been hematologic toxicity, especially myelosuppression. This finding raised concern that use of lenalidomide could adversely affect the ability to mobilize and collect adequate numbers of CD34+ cells for ASCT. In 2 large studies from Mayo Clinic and M.D. Anderson, the most significant factor influencing the ability to collect adequate numbers of stem cells appeared to be initial therapy with lenalidomide (Table 1). In addition to lenalidomide therapy, other important factors impacting the stem cell collection appeared to be the patient age and the duration of lenalidomide therapy. The failure rate of mobilization following lenalidomide therapy has varied significantly between the different studies, likely a reflection of the lenalidomide treatment duration, age of the patient population undergoing stem cell collection, mobilization regimens, and collection targets used. However, data so far do not indicate any impact on the quality of stem cells collected, as reflected in the engraftment kinetics as well as success.

The effect of bortezomib on the ability to collect stem cells has also been examined in the context of phase 2 and 3 trials examining the combinations (Table 1). Although no definite impact of initial therapy on stem cell harvest was demonstrated in the smaller phase 2 studies of bortezomib and dexamethasone, in the IFM 2005/01 trial comparing bortezomib/dexamethasone to VAD there was a trend toward lower CD34+ numbers among those receiving bortezomib. In contrast, in the HOVON-65/GMMG-HD4 randomized phase 3 trial comparing bortezomib, adriamycin, dexamethasone (PAD) versus VAD as induction treatment, no impact of the regimen was seen on the ability to collect stem cells. No significant impact of initial therapy has been seen in other trials that have combined the novel agents, bortezomib in combination with either lenalidomide or thalidomide. Addition of alkylating agents to the initial therapy, especially in combination, may increase the risk of collection failures, but no comparative data are available.

In a phase 2 study looking at the combination of lenalidomide with cytoxan and dexamethasone for newly diagnosed myeloma, we observed 8 of 30 failures at mobilization. In contrast, in a phase 2 study of cytoxan, bortezomib, and dexamethasone by Reeder et al, all patients who attempted stem cell collection were able to get enough cells for at least one transplantation.

Although there has been a wide spectrum of reported data on the initial therapy with a novel agent and the ability to collect stem cells, some common themes have emerged. In the 2 larger experiences published to date of lenalidomide therapy prior to harvest, the number of cycles of therapy appears to be important. Although none of the patients with fewer than 6 cycles of lenalidomide failed to collect stem cells in the Mayo Clinic series, more than 3 cycles of lenalidomide were associated with a higher risk in the M.D. Anderson series. However the smaller studies have not demonstrated such a relationship, and in the absence of detailed data from the larger prospective studies, it would be reasonable to assume that longer duration of therapy will increase the risk of failure. Another common finding has been the age of the patients, with more than one study demonstrating increased likelihood of failure in the older patients. In these 2 studies, no relationship was noted between the time off lenalidomide prior to stem cell harvest. Another important finding across the studies has been the low incidence of collection failure among patients mobilized with chemotherapy, typically cyclophosphamide, and G-CSF. Among 28 treatment-naive patients treated with the combination of clarithromycin, lenalidomide, and dexamethasone (BIRD) reported by Mark et al, sufficient stem cells for 2 autologous stem cell transplants were collected from all patients mobilized with CTX plus G-CSF, versus 33% mobilized with G-CSF alone, demonstrating that this approach can potentially overcome the impairment in stem cell mobilization associated with lenalidomide. For patients failing initial attempts at stem cell mobilization with G-CSF alone, chemotherapy + G-CSF approach appears to have a reasonable efficacy. Five of 7 patients failing G-CSF alone were successfully mobilized with CTX + G-CSF in a study and 18 of 21 patients were remobilized successfully with a chemotherapy + G-CSF approach in another study. Maazumder et al also reported 3 patients who failed to collect successfully despite undergoing mobilization with the CXCR4 inhibitor plerixafor, but were subsequently collected using a combination of cyclophosphamide and G-CSF.

Potential mechanisms of the impact of lenalidomide treatment on stem cell collection

The exact mechanism why lenalidomide inhibits stem cell mobilization is not clear. Koh et al investigated the effects of lenalidomide, pomalidomide (CC4047) (IMiDs), and thalidomide on CD34+ hematopoietic progenitors. They showed in human colony formation assays and long-term culture-initiating cell tests that IMiDs and thalidomide are not toxic to hematopoietic stem cells and do not inhibit self-renewal capacity of stem cells. This makes it less likely that a direct toxic effect of lenalidomide on hematopoietic progenitors explains the limitation in stem cell collection. The group further showed that IMiDs promote myelopoesis with a concomitant maturation stop of neutrophil granulocytes by down-regulation of critical transcription factors such as PU.1. This leads to an accumulation of immature granulocytes within the bone marrow compartment and neutropenia in the peripheral blood. Interestingly, the group also observed that the G-CSF secretion is highly up-regulated in cultures of hematopoietic progenitors treated with IMiDs (day 3 of treatment: control 140 pg/mL, lenalidomide 800 pg/mL, CC4047 1500 pg/mL) (S.L., written communication, September 2008). The biologic reason for the strong up-regulation of G-CSF is unknown. It is likely that self-regulatory mechanisms up-regulate G-CSF to overcome the maturation stop of granulocytes. Higher levels of G-CSF might lead to a tachyphylactic response, resulting in resistance to G-CSF mobilization. These findings are also supported by our observation that all other "non-G-CSF-based" mobilization approaches such as CTX and AMD3100 are successful in mobilizing a sufficient CD34+ cell number.

Suggested approach to stem cell collection in patients undergoing initial therapy with novel agents

In June 2008, a panel of experts was convened by the International Myeloma Foundation to address issues regarding stem cell collection for autologous transplantation in patients receiving therapy
with lenalidomide. The following statements reflect the considerations of the panel and the consensus recommendations formulated by the panel. The recommendations take into account the existing data suggesting compromised cell mobilization with the newer agents in some of the patients as well as the data, although limited, examining alternate approaches to stem cell mobilization. These recommendations will be revised when additional data become available, enabling us to make more specific recommendations.

First attempt: Given the potential for the novel agents to impact the ability of the stem cell collection, we recommend early stem cell mobilization when SCT is being contemplated immediately or later in the course of disease. Such an approach, after 3 to 4 cycles of initial therapy, is quite feasible given the rapid response seen with the new combinations. However, there exists considerable confusion at this point in terms of the mechanisms mediating the decreased collection as well as the best approaches to prevent this problem, and every effort should be made to enroll these patients in clinical trials evaluating these questions.

Among patients undergoing initial therapy with thalidomide or bortezomib in combination with dexamethasone or among those treated with lenalidomide and dexamethasone who have received fewer than 4 cycles of therapy and are younger than 65 years, G-CSF alone is considered adequate for the initial attempt at mobilizing stem cells, although many centers will continue to use cyclophosphamide and G-CSF as their standard protocol. Among those who have received more than 4 cycles of lenalidomide therapy, one should consider the initial use of cyclophosphamide and G-CSF for mobilization. This suggestion is based on the findings of increased failure risk in this population as well as the reduced risk of failure associated with the use of cyclophosphamide and G-CSF. Although the use of cyclophosphamide in all patients is likely to decrease the risk of failure at first attempt, the recommendation to use G-CSF alone in the former group is driven by the low risk of failure in that group, the increased risks and delay associated with use of cyclophosphamide, and finally the ability to successfully collect with cyclophosphamide and G-CSF in the few patients who fail the initial attempt with G-CSF alone. In patients older than 65 years old, we recommend consideration of reduced-dose cyclophosphamide with G-CSF or G-CSF alone with addition of AMD-3100 before the second lenalidomide if the first lenalidomide results in less than 2 million CD34+ cells/kg. In patients receiving other myelosuppressive drugs in combination with lenalidomide, cyclophosphamide and G-CSF should be considered for the initial attempt as the rate of failure increases in these situations. There are no data supporting additional time off therapy prior to mobilization enhancing the likelihood of successful mobilization. We do not recommend a minimum period that patients have to be off lenalidomide prior to starting G-CSF for mobilization.

Failed stem cell collection

Among patients receiving initial therapy with lenalidomide-containing regimens failing to collect with G-CSF alone, there are 3 options for the subsequent attempt. The majority of the patients can be collected with cyclophosphamide priming and G-CSF. These patients will be candidates for use of AMD-3100, which in combination with G-CSF has been very successful in mobilizing stem cells. Another approach includes the use of a combination of G-CSF and granulocyte-macrophage colony-stimulating factor (10 μg/kg per day subcutaneously for 2 days, followed by G-CSF 16 μg/kg per day subcutaneously until stem cell collection is complete).

Upfront use of plerixafor (AMD3100) in lenalidomide-treated patients

The panel discussed the question of routine use of plerixafor for mobilization in this patient group. It was felt that at this time, without a detailed cost benefit analysis, such a recommendation cannot be made and additional clinical trials specifically addressing its use in these patients will allow us to answer this question. Prospective trials should be conducted to study the use of plerixafor in patients failing to reach certain thresholds for peripheral blood CD34 counts.

Authorship

Conflict-of-interest disclosure: S.G. is on the Advisory Board for Celgene, Millennium, Novartis, and Genzyme; E.A.S. is on the Advisory Board for Genzyme; J.L.H. received Honoraria from Genzyme and Amgen, and is on the Advisory Board for Celgene and Janssen-Cilag; A.P. is on the Advisory Board for Ortho Biotech and Celgene; W.B. is on the Advisory Board for Celgene and Millennium, and received research funding from Genzyme, Millennium, Celgene, AstraZeneca, and Novartis; R.L.C. is on the Advisory Board for Millennium and Ortho Biotech; S.K. received clinical trial funding from Celgene, Millennium, and Genzyme; N.M. is on the Advisory Board for Celgene; R.N. received clinical trial funding from Celgene; J.S.M. is on the Advisory Board for Millennium, Janssen-Cilag, and Celgene; H.L. received clinical trial funding from Schering-Plough and Janssen-Cilag, and participated in the Speaker’s Bureau for Amgen, Roche, and Janssen-Cilag; J.B. received honorarium for lectures, is on the Advisory Board for Celgene and Janssen-Cilag, and received a research grant from Celgene; S. Lonial is a consultant for Millennium, Celgene, Novartis, and BMS, H.E. is on the advisory Board for Celgene and Ortho Biotech; P.S. is on the Advisory Board for Ortho Biotech and Celgene; O.S. received clinical trial/research funding from Janssen-Cilag, Merck, and Novartis, and is on the Speaker’s Bureau for Amgen, Celgene, Merck, Novartis, Ortho Biotech, Pharmion, and Roche; and P.G.R. and B.G.M.D. are on the Advisory Board for Celgene and Millennium. P.T., M.C., and S.V.R. declare no competing financial interests.

A complete list of IMWG participants can be found in the supplemental Appendix, available on the Blood website; see the Supplemental Materials link at the top of the online article.

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Mobilization in myeloma revisited: IMWG consensus perspectives on stem cell collection following initial therapy with thalidomide-, lenalidomide-, or bortezomib-containing regimens

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International myeloma working group consensus statement and guidelines regarding the current role of imaging techniques in the diagnosis and monitoring of multiple Myeloma

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Several imaging technologies are used for the diagnosis and management of patients with multiple myeloma (MM). Conventional radiography, computed tomography (CT), magnetic resonance imaging (MRI) and nuclear medicine imaging are all used in an attempt to better clarify the extent of bone disease and soft tissue disease in MM. This review summarizes all available data in the literature and provides recommendations for the use of each of the technologies. Conventional radiography still remains the 'gold standard' of the staging procedure of newly diagnosed and relapsed myeloma patients. MRI gives information complementary to skeletal survey and is recommended in MM patients with normal conventional radiography and in all patients with an apparently solitary plasmacytoma of bone. Urgent MRI or CT (if MRI is not available) is the diagnostic procedure of choice to assess suspected cord compression. Bone scintigraphy has no role in the routine staging of myeloma, whereas sequential dual-energy X-ray absorptiometry scans are not recommended. Positron emission tomography/CT or MIBI imaging are also not recommended for routine use in the management of myeloma patients, although both techniques may be used in selected cases that warrant clarification of previous imaging findings, but such an approach should ideally be made within the context of a clinical trial.

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Keywords: multiple myeloma; conventional radiography; computed tomography; magnetic resonance imaging; nuclear medicine imaging

Introduction

Multiple myeloma (MM) is a plasma-cell malignancy and is characterized by the presence of lytic bone disease causing severe bone pain, pathological fractures, spinal cord compression and hypercalcaemia. Up to 90% of myeloma patients develop osteolytic lesions during the course of their disease. These lesions occur predominantly in the axial skeleton, that is, skull, spine, rib cage and pelvis, as well as the proximal areas of the arms and legs. Furthermore, almost 10% of the patients present with diffuse osteopenia or osteoporosis at diagnosis. Myeloma bone destruction represents a major cause of morbidity and mortality. Progression of skeletal disease is often not affected by chemotherapy even in responding patients. The mechanisms of bone destruction are related to increased osteoclastic bone resorption, which is accompanied by an exhausted osteoblast function and reduced bone formation. Thus, a characteristic feature of myeloma bone disease is that the lesions rarely heal even when the patients are in complete remission. This finding is in keeping with the observation that bone scans are often negative in myeloma patients who have extensive lytic lesions, and offer very little in the follow-up of bone disease in these patients. Appropriate use of imaging techniques is essential in the identification and characterization of the skeletal complications resulting from MM and in determination of the extent of intramedullary bone disease. Imaging also is critical for detection of extramedullary foci, identification and characterization of infections and other complications and evaluation of progression of the disease. However, we lack a consensus and standardized imaging protocol for both newly diagnosed myeloma patients or for following patients in the course of treatment and disease progression.

Lytic lesions are generally diagnosed by radiographic analysis. One weakness of radiographic detection is that it may reveal lytic disease only when over 30% of the trabecular bone has been lost. This results in suboptimal assessment of generalized osteoporosis, which affects MM patients and correlates with an increased risk of early vertebral collapse. The morbidity of vertebral collapse is significant. Chronic pain, functional limitations and respiratory compromise, which increase the risk of pulmonary infections are typical clinical sequelae of vertebral compression fractures. Due to the limitations of standard radiographic analysis, computed tomography (CT) or magnetic resonance imaging (MRI) have been used to increase the sensitivity and specificity of early detection of MM-associated bone destruction. CT and MRI also allow discrimination of malignant and benign compression fractures, visualization of soft tissue involvement and spinal cord and/or nerve root compression or jeopardy.

In recent years, positron emission tomography (PET) has also been used in MM imaging. 18F-fluorodeoxyglucose (FDG) is taken up by metabolically active cells, which can then be imaged using PET. High uptake by tumor cells is visible on PET.
Role of Imaging techniques in multiple myeloma
M. Efroymson et al

imaging, as they have increased metabolic rates. This review summarizes all available data for the role of imaging in MM and aims to provide practical information for the usage of these techniques by clinicians who manage myeloma patients.

Conventional radiology

Since 1903, when Weber first observed that myeloma lesions are evident on radiographs, X-rays have been extensively used to identify myeloma-related bone lesions both at diagnosis and during disease course. Lytic lesions on plain X-rays are typically holes—that is, punched-out lesions with absent reactive sclerosis of the surrounding bone—in the flat bones of the skull and pelvis. In the long bones, there is a range of appearances from endosteal scalloping to discrete small (<1 cm) lytic lesions, to mottled areas of multiple small lesions, to large destructive lesions. These lesions correspond to nodular replacement of marrow by plasma cells with entire bone destruction. Conventional radiography may also reveal diffuse osteoporosis, which is best recognized in the spine.

The presence of lytic lesions is a criterion for myeloma diagnosis, whereas the extent of lytic disease is included in Durie-Salmon staging system. Therefore, it is important to include in a 'complete skeletal survey' all areas of possible myeloma involvement, such as the cervical, thoracic and lumbar spine, skull, chest, pelvis, humeri and femora. Almost 80% of patients with myeloma will have radiological evidence of skeletal involvement on the skeletal survey, most commonly affecting the following sites: vertebrae in 65% of patients, ribs in 45%, skull in 40%, shoulders in 40%, pelvis in 30% and long bones in 25%. However, radiologically detectable lesions distal to the elbows and knees are exceptional. Patients who are asymptomatic but have radiological evidence of bone disease (at least one lytic lesion) are at high risk of progression with a median time to progression of 8 months. The importance of the presence of lytic lesions is further supported by the notion that in the International Myeloma Working Group Classification for plasma cell dyscrasias, patients with bone disease are classified as 'symptomatic' and require treatment even in the absence of clinical symptoms.

However, even with complete radiographic surveys 10–20% of the patients have normal results. This may be due to some important disadvantages of conventional radiology, as suggested in Table 1. In plain X-rays some areas are not well visualized; for this reason both lateral and anteroposterior views of the spine are needed for the better visualization of the vertebral bodies. Furthermore, conventional X-rays have limited sensitivity as they cannot detect early lytic lesions and limited specificity as they fail to distinguish myeloma-related osteoporosis from osteoporosis due to other reasons, such as steroid-induced or postmenopausal osteoporosis. The observer and technology dependence of conventional X-rays have also the risk of underdiagnosis of lytic disease. It has also reported that the reproducibility of the results is very low between different centers and in a recent study an expert radiological review of skeletal surveys was able to detect additional abnormalities in 23% of the studied cases. A major disadvantage of conventional X-rays is that almost 20 separate films/exposures are needed, requiring a lengthy period on the radiographic table. The patient's ability to tolerate the standard bone survey is an important issue because myeloma patients can experience severe pain when they are rotated and positioned for multiple individual radiographic exposures. To override this problem, some centers have introduced a whole-body conventional radiographic skeletal survey, the low-dose whole-body radiographic system (Statixcan) for the detection of focal metastatic deposits in cancer and myeloma patients, which can give a high quality imaging of the bones in less than 5 min. In a study of 30 patients with solid tumors metastatic to the skeleton and MM, the whole-body radiography was found as effective as CT or MRI in revealing focal lesions, a result that has not yet been confirmed by others. Furthermore, plain X-rays cannot be used for the assessment of response to therapy as the lytic bone lesions seldom show evidence of healing whereas new compression vertebral fractures do not always indicate disease progression and may occur due to ongoing bone loss or reduction of tumor mass that supports the bony cortex. For all these reasons, although conventional X-rays are considered as a 'gold standard' for the determination of the extent of myeloma bone disease at diagnosis, further imaging is needed during follow-up mainly in the absence of the detection of lytic lesions or the presence of diffuse osteoporosis only.

Computed tomography

CT scanning allows the detection of small osteolytic lesions in MM, which are not revealed by plain radiography. CT imaging is much faster than standard radiographic procedures and allows excellent 3D reconstruction of images. In a few institutions, CT scanning has replaced conventional radiography as the initial imaging tool used in patients with trauma to the spine or pelvis. Furthermore, CT can accurately depict the extent of associated soft tissue masses and can direct needle biopsy for histological diagnosis. The advantages of CT vs conventional X-rays: (1) the duration of the examination is practically three times less than that necessary to perform standard radiography; therefore, there is significant economy in the work time of the technicians; (2) CT scanning allows the complete diagnostic evaluation in a single examination without having to reposition the patients, a procedure that is necessary in conventional studies; this is certainly an important point to consider when examining a patient in pain; (3) the diagnostic sensitivity of CT imaging is superior to that of standard radiography and reveals more lesions as compared with conventional radiology, mainly in areas that cannot be accurately visualized by plain radiography, for example, scapulae, rib or sternum; (4) CT has proven to be superior in estimating fracture risk and instability; (5) CT scanning can demonstrate other underlying pathological processes, especially those involving the lungs, although the percentage is not significant; (6) it is superior in planning the radiation therapy or the surgical intervention as it depicts the anatomic area very accurately (Table 2). Furthermore, a novel CT technique, the multidetector row computed tomography (MDCT) was found to be very sensitive in detecting small osteolytic lesions (<5 mm) in the spine, as compared with MRI and PET.

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<th>Table 1</th>
<th>Conventional radiology: Limitations</th>
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<td>• Some areas not well visualized</td>
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<td>• Limited sensitivity: 10–20% of lesions/abnormalities missed</td>
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<td>• Reduced specificity vs benign causes of osteoporosis</td>
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<td>• Observer dependent</td>
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<td>• Time/tolerance for standard survey not ideal</td>
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<td>• Usual fail to show response to treatment</td>
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Table 2 Advantages of computed tomography (CT)

- Detects small osteolytic lesions
- Faster than standard radiographic survey
- Provides 3D reconstruction of images
- Shows associated soft tissue disease
- Greater sensitivity and specificity versus standard radiography
- Allows estimation of fracture risk
- Excellent for radiotherapy planning and for surgical intervention

Table 3 Role of magnetic resonance imaging (MRI)

- More sensitive than standard radiography
- Excellent imaging of axial skeleton
- Discriminates myeloma vs normal marrow
- Excellent diagnostic discrimination for spinal cord/nerve compression issues, as well as soft tissue disease
- Can detect avascular necrosis of the femoral head
- Can detect arthrolytic joint chain deposits in the heart and other sites
- Can be used to assess disease status in monoclonal gammopathy of undetermined significance (MGS), asymptomatic myeloma and for solitary plasmacytoma of bone
- Can be used to monitor response (although improvements can be delayed)

One of the negative points advanced against CT scanning is the radiation dose delivered to patients. The amount of radiation is 1.3-3 times higher than that delivered during standard radiography.31,34 In summary, conventional or low-dose CT scanning of the spine is considered to be a realistic alternative to standard radiography in MM patients presenting with painful symptoms because it allows for obtaining an exhaustive evaluation of the skeletal lesions in a short period. Furthermore, CT is helpful as a basis for radiation therapy planning, for the preparation for surgical intervention to delineate the anatomic architecture as precisely as possible and for a CT-guided needle biopsy. Finally, CT may identify lesions that are negative on plain radiography, and should be considered in patients who remain symptomatic despite having no evidence of osteolysis on the skeletal survey.

Magnetic resonance imaging

MRI has been widely available for the evaluation of MM during the last two decades and is used by several myeloma centers of excellence for the management of myeloma patients. MRI allows visualization of the medullary cavity and a direct assessment of the degree of MM cell infiltration before bone destruction becomes visible on plain radiographs, in the absence of radiation exposure.29,36 Furthermore, in the event of suspected cord compression, MRI is the technique of choice.37 It provides an accurate assessment of the level and extent of cord or nerve root compression, the size of the tumor mass and the degree to which it has extended into the epidural space. MRI can also be used to predict the risk of vertebral fracture. Patients with advanced myeloma who had more than 10 lesions on spinal MRI had a 6- to 10-fold higher risk of fracture than patients who had normal appearance or fewer than 10 lesions on MRI.38 However, MRI does not predict the risk of fracture by level.59

MRI can assist in the distinction between benign from malignant compression fractures. A benign osteoporotic fracture is suggested when a retrospursed bone fragment is seen, when fat signal is preserved on T1-weighted images throughout the body and there is no high signal on T2-weighted images, when there is only a thin (<1 cm) surrounding soft tissue component and when horizontal band-like areas representing the fracture plane are seen following gadolinium administration. A malignant etiology of collapse is suggested when the posterior cortex is convex toward the spinal canal, epidural mass is seen, when the entire vertebral body or pedicles are replaced by low signal on T1-weighted images, and high or heterogeneous signal is seen within the body following gadolinium injection or on T2-weighted images.10 MRI can be also used for the accurate illustration of the vertebral fractures or the percentage loss of vertebral height before the performance of percutaneous vertebroplasty and kyphoplasty.80,81

MRI imaging is the most sensitive and specific imaging modality for the diagnosis of avascular necrosis of the femoral head that may result from high-dose steroid therapy or radiotherapy, and is demonstrated by the presence of the characteristic double-line sign on T2-weighted MR images.42 Early recognition of avascular necrosis before the development of a subchondral fracture is extremely important for the success of conservative management.

In general, the advantages of MRI over conventional radiography and CT scan include: (1) the excellent imaging of the axial skeleton due to the greater sensitivity of the method, (2) the discrimination of myeloma from normal marrow, (3) the accurate illustration of spinal cord and/or nerve root compression, soft tissue extension, head and neckplasmacytomas, avascular necrosis of the femoral head and (4) better evaluation of cardiac amyloidosis and/or soft tissue amyloid deposits (see Table 3).

MRI sequences in myeloma

Several MRI techniques have been developed to aid in the assessment of the bone marrow in hematological malignancies.43 The MRI sequences that are most informative are the T1-weighted, the T2-weighted with fat suppression, the short time inversion recovery (STIR) and the gadolinium T1-weighted with fat suppression. Typical myeloma lesions have a low signal intensity on T1-weighted images and a high signal intensity on T2-weighted and STIR images44 and generally show enhancement on gadolinium enhanced images. In a recent study, three MRI sequences were evaluated to reveal the method which provides the highest confidence level in depicting the MM lesions.45 The authors compared a precontrast T1w-TE sequence (TR: 700 ms, TE: 10 ms), a T2w-TIRM sequence (TR: 8000 ms, TE: 80 ms) and a contrast-enhanced T1w-TSE sequence with fat saturation (TR: 700 ms, TE: 10 ms). The turbo inversion recovery magnitude (TIRM) sequence is a turbo spin-echo sequence (TSE) with an inversion recovery pulse (IR) in combination with the calculation of the magnitude signal intensity (M). Studying 59 MRI examinations of 23 consecutive patients, the authors found that the T2w-TIRM sequences achieved the highest level of sensitivity and best reliability. However, they suggest that for an exact staging and grading the examination protocol should encompass unenhanced and enhanced T1w-MRI sequences, in addition to T2w-TIRM.45

MRI patterns in myeloma

Five MRI imaging patterns of marrow involvement in myeloma have been recognized: (1) normal appearance of bone marrow despite moderate microscopic plasma cell infiltration, (2) focal involvement, (3) homogeneous diffuse infiltration, (4) combined...
diffuse and focal infiltration, (S) 'salt-and-pepper'-pattern with inhomogeneous bone marrow with interposition of fat islands.\textsuperscript{35,46} In almost 30\% of MM patients a normal-looking bone marrow signal is found in all sequences with high signal on T1-weighted and intermediate signal intensity on T2-weighted spin-echo images as well as low signal in fat-saturated sequences, such as STIR.\textsuperscript{35} More specifically, a normal marrow appearance is present at diagnosis in 30-75\% of untreated Durie-Salmon stage I myeloma and in 20-30\% of untreated Durie-Salmon stage II disease.\textsuperscript{46-48} In histology, this corresponds to a slight interstitial plasma cell infiltration (<0.2\% vol\% in bone marrow biopsy).

The focal pattern consists of localized areas of abnormal marrow and is found in approximately 30\% of myeloma cases. On T1-weighted images, focal lesions are darker that yellow marrow and slightly darker or iso intense to red marrow. On T2-weighted images they are brighter than both red and yellow marrow, and on enhanced T1-weighted images they enhance with various degrees depending on the vascularity of the underlying myeloma. STIR and fat-saturation T2-weighted images provide contrast between focal lesions and uninvolved marrow.\textsuperscript{35,47}

In the diffuse MR pattern of abnormal marrow, the normal bone marrow is completely replaced by the abnormal process. The intervertebral discs appear brighter or iso intense to the diseased marrow. On T1-weighted images, there is a diffuse decrease in the signal intensity of the marrow. On T2-weighted images, a variable increase in the signal intensity of the abnormal marrow is observed. After the administration of intravenous contrast, the abnormal marrow enhances. The intervertebral discs appear darker than the enhanced spine.\textsuperscript{35,47}

A combined focal and diffuse infiltration pattern can be found in about 10\% of myeloma patients. On T1-weighted SE images the bone marrow signal intensity is diffusely decreased with additional foci interspersed. Those foci are often better demarked on fat-saturated or gradient-echo images.

Finally in about 3-5\% of the patients the so-called 'salt-and-pepper'-pattern can be found. On T1-weighted SE images, and also on gradient-echo and T2-weighted SE sequences, the bone marrow presents a very inhomogeneous patchy pattern. However, no hyperintense areas are demarcated in fat-saturated sequences. This imaging corresponds to bone marrow with circumscribed fat islands beside normal bone marrow with a minor infiltration of plasma cells (<20\%).\textsuperscript{35-49}

Low tumor burden is usually associated with a normal MRI pattern, but a high tumor burden is usually suspected when there is diffuse hypointense change on T1-weighted images, diffuse hyperintensity on T2-weighted images and enhancement with gadolinium injection. In general, patients with normal or 'salt-and-pepper' MRI pattern tend to have signs of lower tumor burden than those with diffuse or focal marrow involvement patterns.\textsuperscript{37-49}

Furthermore, a significant correlation between diffuse and focal MRI patterns of marrow involvement with low serum hemoglobin values and high percentage of marrow plasmacytosis has been reported, suggesting that diffuse or focal marrow involvement patterns correlate with high tumor burden.\textsuperscript{47}

The main methodological consideration with MRI imaging is the lack of specificity of the findings. Focal or diffuse changes may exist at diagnosis, may be variations of the normal, or reflect an alternative pathological or physiological process such as iron loading,\textsuperscript{50-51} amyloid deposition \textsuperscript{51} or reactive marrow hyperplasia.

MRI findings in MGUS
Monoclonal gammapathy of undetermined significance (MGUS) is defined by a monoclonal immunoglobulin concentration in serum of 3 g/100 mL or less, the absence of lytic bone lesions, anemia, hypercalcemia and renal insufficiency related to the proliferation of monoclonal plasma cells, and a proportion of plasma cells in the bone marrow of 10\% or less. In large referral centers, half the patients with a monoclonal gammapathy have MGUS, whereas only 15\% to 20\% have MM.\textsuperscript{54} Although, lytic lesions are not found in MGUS by definition, osteoporosis is a common finding among MGUS patients who have a higher incidence of vertebral fractures compared to normal population.\textsuperscript{59} Therefore, sometimes it is difficult to differentiate MGUS from early myeloma. MRI studies have been performed in patients with MGUS. Belloaiche et al found that the MRI of the thoracolumbar spine was normal in all tested patients with MGUS (n = 24) compared with only 6 out of 44 (13.6\%) with newly diagnosed MM.\textsuperscript{60} In another study, bone marrow abnormalities were detected with MRI imaging in 7 out of 37 patients (19\%) with MGUS or monoclonal gammapathy of borderline significance (all MGUS criteria but plasma cell infiltration of between 10 and 30\%). All patients with a normal MRI investigation had not required treatment after a median follow-up of 30 months, whereas time to progression to MM was significantly higher for patients with abnormal MRI.\textsuperscript{51}

MRI and solitary plasmacytoma of the bone
Approximately 2\% of patients with plasma cell dyscrasias have solitary bone plasmacytoma (SBP). The diagnosis of SBP requires a solitary bone lesion, a biopsy of which shows infiltration by plasma cells, negative results on a skeletal survey, absence of clonal plasma cells in a random sample of bone marrow and no
evidence of anemia, hypercalcemia or renal involvement suggesting systemic myeloma. Although definitive radiotherapy usually eradicates the local disease, the majority of patients will develop MM because of the growth of previously occult lesions which have not been detected by conventional radiography. MRI imaging is the preferred imaging modality for the initial assessment and for the follow-up of the osseous and extraneous extent of an SBP. Moulopoulos et al showed that MRI of the thoracic and lumbarosacral spine showed additional foci of marrow replacement in four of 12 patients with SBP; thus some patients who have an SBP diagnosed by standard criteria may be understaged if an MRI is not performed. After treatment with definitive radiotherapy to the painful lesion, three patients developed systemic disease within 18 months from diagnosis. Furthermore, Liebross et al reported that among SBP patients with thoracolumbar spine disease, seven of eight staged with plain radiographs alone developed MM in comparison with only one of seven patients who also had MRI studies of the spine. These results suggest that MRI should be part of the staging procedures in patients with SBP, to better assess both the extent of the local tumor and the revealing of occult lesions elsewhere. Coronal images of the central skeleton may increase the incidence of unsuspected lesions.

**MRI in smoldering multiple myeloma**

Asymptomatic patients with paraprotein level in the serum of >30 g/l and/or bone marrow clonal plasma cells of >10%, and no myeloma-related organ or tissue impairment, are considered to have smoldering multiple myeloma (SMM), according to the International Myeloma Working Group.21 These patients account for about 15–20% of myeloma patients, and have a median time to disease progression of 2–3 years. According to current practice, patients with SMM may remain stable for years without therapy and thus should be followed without treatment until there is evidence of imminent disease progression. Asymptomatic patients with at least one lytic lesion in conventional X-rays have a median time to progression of 10 months; therefore, they should be treated at diagnosis. MRI reveals abnormal marrow appearance in 30–50% of the patients. Moulopoulos et al reported that patients with abnormal MRI studies required therapy after a median of 16 months vs 43 months for those with normal MRI studies (P<0.01). Moreover, Mariette et al showed that during a median follow-up of 25 months, 10 out of 53 SMM patients developed disease progression; of those, 8 out of 17 had abnormal MRI and 2 out of 38 patients had normal MRI. In that study, abnormal MRI independently predicted for time to progression. This result has not been confirmed by other studies. However, MRI may be particularly useful in patients with asymptomatic myeloma who have an intermediate risk for disease progression.

**MRI and assessment of response**

MRI can be used to assess the effects of antmyeloma therapy, although the response rates to conventional chemotherapy are similar among patients with different MRI patterns and the time to complete response (CR) is similar among patients with different number of focal lesions on MRI (>7 vs ≤7). A change in MRI pattern may correlate with response to therapy. Moulopoulos et al reported that CR is characterized by complete resolution of the preceding marrow abnormality, and partial response is demonstrated by conversion of a diffuse to a variegated or focal pattern. Features suggestive of an objective response to treatment include a reduction in signal intensity on T2-weighted spin-echo images and the absence of contrast-induced rim-enhancement that was previously present. Focal lesions may shrink or remain unchanged in size after effective antmyeloma therapy or they may remain hyperintense in both responders and nonresponders to treatment due to treatment-induced necrosis and inflammation. Therefore, post-antmyeloma therapy MRI of the bone marrow may provide important information for patients with equivocal clinical and laboratory results as well as for patients with nonsecretory myeloma. In a study by the Arkansas group, focal lesions were present on MRI in 27 of 30 patients with nonsecretory MM. After treatment, bone marrow-defined CR occurred in 22 (81%) of these 27 patients, and MRI-CR was documented in 41% of patients at 36 months.

Autologous stem cell transplantation (ASCT) is considered the treatment of choice for younger myeloma patients. Lecouvet et al developed an index for the assessment of changes occurring in the spine after transplant. The index numerically combines findings related to the number of lesions, lesion size, contrast enhancement and marrow background. A score of 0, 1 or 2 is given for each parameter depending on whether there is improvement, stability or worsening. Patients with an index below 4 had a better treatment response than those with an index of 4 or more. In this point, it is crucial to mention that MRI evaluation post-ASCT has to be performed at least 1 month after G-CSF administration. There can be diffuse or focal marrow changes after treatment with G-CSF that cannot be easily distinguished by active disease.

**MRI findings and prognosis in symptomatic myeloma**

The prognostic value of MRI findings in symptomatic myeloma has been evaluated in different studies. Patients with a single lytic lesion on plain radiography, who are found to have further lesions on MRI have a shorter time to progression and shorter time to starting therapy compared to those with a normal MRI study. Patients with advanced disease who have normal MR findings and receive conventional dose chemotherapy have a longer survival compared to those with diffuse or focal abnormalities on MRI imaging. The pattern of MR bone marrow involvement in myeloma also has prognostic significance, with both focal and diffuse patterns being associated with a higher tumor burden. In 142 symptomatic myeloma patients, Moulopoulos et al showed that the median survival was 24 months for patients with the diffuse pattern, 51 months for those with the focal pattern, 52 months for those with the variegated pattern and 56 months for patients with the normal pattern (P=0.001). The presence or absence of a diffuse MRI pattern separated patients with ISS stages I and II into two subgroups with significantly different survival times of 28 months and 61 months, respectively (P=0.01). Furthermore, a diffuse MRI pattern predicted inferior outcome regardless of whether or not patients had received high-dose therapy with ASCT.

The largest study in the literature, which reported on the prognostic value of MRI in myeloma patients was published by the Arkansas group. In 611 myeloma patients who were treated uniformly with a tandem autologous transplantation-based protocol, MRI, but not conventional radiography, defined focal lesions independently affected survival. In particular, cytogenetic abnormalities and more than seven focal lesions on MRI distinguished three risk groups: 5-year survival was 76% in the absence of both more than seven focal lesions on MRI and cytogenetic abnormality (n = 276), 61% in the presence of one
of these adverse features (n = 262) and 37% in the presence of both unfavorable parameters (n = 67). High number of MRI focal lesions (> 7) correlated with low albumin and elevated levels of C-reactive protein, lactate dehydrogenase and creatinine, but did not correlate with age, β2-microglobulin and cytogenetic abnormalities. Resolution of the focal lesions on MRI post-antimyeloma therapy that occurred in 60% of the patients identified a subgroup with superior survival. Furthermore, at disease progression after CR, according to clinical criteria, MRI focal lesions were present in 70% of the patients, including 26% with new focal lesions outside of the areas of initial involvement, 28% focal lesions that were larger than the original lesions and 15% with both an increase in original size and new MRI focal lesions.57

**Nuclear medicine imaging**

Traditional technetium bone scintigraphy has high sensitivity for the detection of solid tumors metastatic to the skeleton but its sensitivity in MM and solitary plasmacytoma is very low. Technetium bone scintigraphy scanning may detect lytic lesions in 35–60% of MM patients, but its specificity and sensitivity at the time of the initial diagnosis, in follow-up studies and in the evaluation of bone pain is lower compared to conventional radiography.78-80 In myeloma patients, the skull, the extremities, the iliac and pubic bones are better assessed with plain radiography, whereas for new vertebral lesions and for lesions in the ribs and sternum, bone scintigraphy seems to be superior and for sacrum both methods are equal.81 The inferiority of bone scans vs conventional radiography is primarily due to the osteolast dysfunction in myeloma.1,4,9-11 as skeletal uptake of 99mTc-diphosphonate is related mainly to osteoelastic process. Therefore, newer techniques have been developed in an effort to improve the sensitivity of detection of myeloma bone disease.

**99mTc-sestamibi**

99mTc-labeled hexakis-2-methoxyisobutylisonitrile (99mTc-sestamibi) is a lipophilic cationic γ-emitting radiopharmaceutical originally introduced as a myocardial perfusion imaging tracer. Because of its biochemical characteristics, which favor accumulation in tissues with high cell density and mitochondrial activation, 99mTc-sestamibi (MIBI) is actively concentrated in a variety of malignant tumors such as sarcomas, breast, brain, lung and thyroid cancers.82 MIBI imaging closely reflects myeloma disease activity in bone marrow with very high sensitivity and specificity.83-84 Additionally, bone marrow MIBI uptake is linearly related to bone marrow biopsy results and MIBI was reported to be localized inside the plasma cells infiltrating the bone marrow.5-67

In MGUS patients, MIBI is always negative81,86,87 and it cannot be used to predict MGUS transformation; thus it is not useful in MGUS work-up.88 MIBI imaging can detect soft and skeletal lesions in MM patients and is more sensitive than conventional radiography.89 Its overall sensitivity is approximately 92% and its specificity is 96%.89 However, MIBI imaging has inferior value compared to FDG-PET/CT,91 and found to underestimate the extent of myelomatous bone marrow infiltration in the spine, especially in patients with low disease stage, compared to MRI.97 The pattern of MIBI uptake is significantly different in MM patients. Focal uptake reflects active myeloma sites, whereas diffuse uptake without the presence of focal uptake does not indicate active myeloma.95 MIBI score was significantly related to ISS, bone marrow biopsy infiltration rate and serum β2-microglobulin.88,94 Furthermore, MIBI washout may predict for response to conventional or high-dose chemotherapy.95,96 MIBI scan added no relevant prognostic information to the ISS in patients with stages I and III MM, but the MIBI scan was of prognostic value in stage II MM patients.98 MIBI scan cannot detect the necrotic lesions of osteonecrosis of the jaw in myeloma patients.97

**Positron emission tomography**

PET is a tomographic nuclear imaging procedure that uses positrons as radiolabels and positron-electron annihilation reaction γ-rays to locate the radiolabels. A low dose of a radiopharmaceutical labeled with a positron emitter, such as 18-fluorine-fluoro-deoxyglucose (FDG), is injected into the patient, who is scanned by a tomographic system. The main limitation of PET scanning is limited spatial resolution; thus subcentimeter lytic lesions seen on plain radiographs may not be detectable on PET scanning.99 The advent of fusion scanning combining both PET and CT addresses the issue of limited spatial resolution. In PET/CT fusion scanning, the patient receives an injection of FDG about 1h before image acquisition. After the patient is positioned on the scanner bed, an initial topogram is acquired to define the examination range for the PET/CT image acquisition (usually from the ears to the hips). A spiral CT is then performed after which the scanner bed is moved back to the starting position and the PET scan commenced. Reconstruction of the image, incorporating PET and CT data are completed soon after PET/CT image acquisition. The actual scanning time is shorter for PET/CT (approximately 30 min) than a PET scan alone (approximately 1h) because CT data are used to perform attenuation correction.99

Several studies have shown PET/CT is reliable for most bone lesions that are at least 1 cm in diameter using a standard SUV cutoff of 2.5 to indicate the presence of disease.100 For lesions smaller than 5 mm in diameter, it has been suggested that any amount of FDG uptake should be considered positive regardless of SUV. Lesions between 5 and 10 mm are considered indeterminate if the SUV is less than 2.5. The patient’s weight and body mass are additional factors that affect the SUV.101 The sensitivity of FDG PET in detecting myelomatous involvement is approximately 85% and its specificity is 92%.98 The first assessment FDG PET in myeloma, a study of 66 patients followed serially, showed that FDG PET allows identification of high-risk myeloma and can be used to monitor nonsecretory myeloma as well as patients in CR without measurable M-component.102 This led to the inclusion of myeloma into larger studies of PET/CT in the United States.103,104 The National Oncologic PET Registry (NOPR), a large prospective program, enrolled 22975 cancer patients in the first year and revealed that 36.5% of the time treating physicians changed the intended management of the basis of PET/CT results. The registry has thus far included over 1300 myeloma patients. PET/CT has been included as an option in the diagnosis and monitoring of myeloma patients within NCCN guidelines.105 Further targeted studies in myeloma are required to further clarify aspects of the specific utility in myeloma patients. In addition to demonstrating persistent or recurrent osseous disease, PET/CT studies are more sensitive than other imaging techniques for localizing extramedullary sites of disease, where they reveal additional lesions in almost 30% of the patients who had been diagnosed with solitary plasmacytoma by MRI.106,107 In two recent studies in patients with SIB, PET/CT allowed detection of other unsuspected sites of bone involvement, upstaging the extent of
the disease and significantly affect the therapeutic decisions. In a prospective comparison among 18F-FDG PET/CT, MRI and conventional radiography (whole-body X-rays) in 46 newly diagnosed myeloma patients, PET/CT was superior to plain radiographs in 46% of patients, including 19% with negative X-rays. However, in 30% of patients, PET/CT scans of the spine and pelvis failed to show abnormal findings in areas in which MRI revealed an abnormal pattern of bone marrow involvement, more frequently of diffuse type. In contrast, in 35% of patients, PET/CT enabled the detection of myelomatous lesions in areas which were out of the field of view of MRI. By combining MRI of the spine/pelvis and PET/CT the ability to detect sites of active MM, both medullary and extramedullary, was as high as 92%. Following ASCT, 15 out of 23 patients had negative PET/CT scans (including 13 with a very good partial response or at least a near CR), but only 8 had normal MRI. There are several small studies supporting that either 18F-FDG PET/CT is comparable to the in the detection of focal lesions in spine and pelvis, but it was superior for an accurate whole-body evaluation or MRI is superior to FDG-PET in detecting bone marrow involvement in the spine of patients with advanced MM. In summary, although all reported studies have confirmed the superiority of PET/CT over conventional radiography, they have also revealed that PET/CT was the sole imaging study done, it would miss many additional small lytic skeletal lesions and could miss diffuse spine involvement compared to MRI. Another disadvantage of PET/CT is the false-positive results it has especially in areas of inflammation or infection, deposits of brown fat (especially in the mediastinum and neck), postsurgical changes, vertebralplasty changes and occasionally other benign or malignant processes, such as renal, pancreatic, uterine and prostate cancer.

FDG PET/CT was found more sensitive than MRI for making the diagnosis of mandibular osteonecrosis although it is not an accurate method for the detection of femoral head osteonecrosis. To override these problems, novel radiolabeled agents have also been used in PET/CT. The use of the radiolabeled amino-acid carbon 11 (11C) methionine with PET/CT showed 11C-methionine-positive lesions in normal cancellous bone in the majority of 19 MM patients, and in all patients with extramedullary disease.

In general, MIBI and PET/CT are useful additional diagnostic tools for detecting otherwise occult sites of myeloma. A recent large study of MIBI on the relative impact of PET on patients with 15 different types of known cancers for three distinct indications (initial staging, restaging and detection of suspected recurrence) revealed that when intended management was classified as treatment or nontreatment, physicians changed their intended management for almost 49% of myeloma cases. This result depicts the change of management of MM patients with the broad use of PET in myeloma. However, further studies are needed before the recommendation of using PET as a standard tool in both diagnosis and follow-up of MM patients. Finally, the use of MIBI PET should particularly be considered in the evaluation of a patient with an early-stage MM to exclude the presence of more extensive disease.

Dual-energy X-ray absorptionometry

Osteoporosis in the general population is currently diagnosed using dual-energy X-ray absorptionometry (DEXA). In MM patients, reduced lumbar spine bone mineral density correlates with increased risk for early vertebral fractures. This makes DEXA a valuable test to consider, as it may also influence the decision to begin bisphosphonate treatment, which can produce a 5–10% improvement over a 6-month period. Another advantage of DEXA is that the technique, which involves assessment of bone mineral density (BMD) in the lumbar spine, hip and distal radius, is a quick, noninvasive investigation that uses a small dose (<1 ISV) of radiation. Disadvantages of the method include its influence by spondylisis, spinal osteophytes and the presence of vertebral collapse, and its difficulty to recognize myeloma osteoporosis from malignant osteoporosis. Furthermore, sequential DEXA-scans shows heterogeneous local BMD changes, and cannot predict disease progression.

Conclusions

Various imaging technologies have been used for the diagnosis and management of myeloma patients. As part of the staging procedure of newly diagnosed myeloma, the skeletal survey is mandatory and should include a posteroanterior view of the chest, anteroposterior and lateral views of the cervical spine (including an open mouth view), thoracic spine, lumbar spine, humeri and femora, anteroposterior and lateral views of the skull and anteroposterior view of the pelvis. In addition, symptomatic areas should also be specifically visualized. Whole-body, low-dose MDCT has substituted conventional radiography in some centers for both diagnosis and follow-up of MM patients and the clinicians have to take this method into consideration if it is available. Whole-body MRI can give complementary information to skeletal survey and is recommended in patients with normal conventional radiography. MRI of the whole spine should be performed in addition to the skeletal survey as part of staging in all patients with an apparently solitary plasmacytoma of bone irrespective of site of index lesion. Urgent MRI is the diagnostic procedure of choice to assess suspected cord compression in myeloma patients even in the absence of vertebral collapse. Urgent CT may be used to establish the presence of suspected cord compression in cases where MR Imaging is unavailable, impossible due to patient intolerance or contraindicated, for example, intraorbital metallic foreign bodies or cardiac pacemakers. CT of the spine or other areas of the skeleton may be considered to clarify the presence or absence of bone destruction in cases of clinical concern. Furthermore, CT is indicated to clarify the nature and extent of soft tissue disease and, where appropriate, to guide tissue biopsy. MRI should be used to clarify the significance of ambiguous CT findings, as these two imaging techniques can give complementary information, whereas both can be used before vertebralplasty or kyphoplasty. Bone scintigraphy has no role in the routine staging of myeloma, although sequential DEXA scans are not recommended. Based on the currently available evidence, neither PET nor MIBI imaging can be recommended for routine use in the management of myeloma patients, although both techniques may be useful in selected cases that warrant clarification of previous imaging findings, but such an approach should ideally be made within the context of a clinical trial.

In the event of disease progression, the skeletal survey should be repeated as part of the restaging process. Any newly symptomatic areas of the skeleton should be specifically targeted. MRI should be performed in all patients with negative skeletal survey. MRI or CT can be used for monitoring the response of soft tissue masses to therapy. The usefulness of PET/CT and MIBI on the follow-up of myeloma has not been
confirmed and further trials are needed. Treating physicians
must keep foremost in mind that myeloma bone disease is often
the cause of the most disabling problems that patients face and,
therefore, careful baseline and serial radiographic assessments
are essential to maintaining and improving their patients' quality
of life.

Conflict of interest

The authors declare no conflict of interest.

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Paul Richardson, Dana Farber Cancer Institute, Boston, MA, USA
Angelina Rodriguez Morales, Conoco Metro Politano de
Sangre, Caracas, Venezuela
Orhan Sezer, Department of Hem/Onc, Universitätsklinikum
Charite, Berlin, Germany
John Shaughnessy, MIRT UAMS, Little Rock, AR, USA
Kazuyuki Shimizu, Nagoya City Midori General Hospital,
Nagoya, Japan
David Siegel, Hackensack, Cancer Center, Hackensack, NJ, USA
Jesus San Miguel, University of Salamanca, Salamanca, Spain
Chaim Shustik, McGill University, Montreal, Canada
Seema Singhal, Northwestern University, Chicago, IL, USA
Pieter Sonneveld, Erasmus MC, Rotterdam, The Netherlands
Andrew Spencer, The Alfred Hospital, Melbourne, Australia
Edward Stadmazer, University of Pennsylvania, Philadelphia, PA, USA
Keith Stewart, Mayo Clinic Arizona, Scottsdale, AZ, USA
Patrizia Tosi, Italian Cooperative Group, Istituto di Ematologia Seragnoli, Bologna, Italy
Guido Tron, Huntsman Cancer Institute, Salt Lake City, UT, USA
Ingermar Turesson, Department of Hematology, Malmo University, Malmo, Sweden
Brian Van Ness, University of Minnesota, Minneapolis, MN, USA
Ivan Van Riet, Brussels Vrije University, Brussels, Belgium
Robert Vesco, Cedars-Sinai Cancer Center, Los Angeles, CA, USA
David Vesole, Loyola University Chicago, IL, USA
Anders Waage, University Hospital, Trondheim, Norway
NSMG Michael Wang, MD Anderson, Houston, TX, USA
Donna Weber, MD Anderson, Houston, TX, USA
Jan Westin, Sahlgrenska University Hospital, Gothenburg, Sweden
Keith Wheatley, University of Birmingham, Birmingham, United Kingdom
Dina B Yehuda, Department of Hematology, Hadassah University Hospital, Hadassah, Israel
Jeffrey Zonder, SWOG, Department of Hem/Onc, Karmanos Cancer Institute, MI, USA

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Leukemia
REVIEW

Immunohematopoietic stem cell transplantation: introduction and 35 years of development in South Africa—the historical and scientific perspective

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Bone marrow was the traditional graft source when we introduced these procedures to South Africa. Technical details were established using rabbits as the experimental model with translation into a formally structured clinical programme at the University of Cape Town, based in the Groote Schuur Hospital, in 1972. Lack of any infrastructure was overcome by the acquisition of the first continuous-flow cell separator in sub-Saharan to provide for granulocyte transfusions. This was shortly followed by creating a dedicated platelet donor panel and establishing a specialized laboratory for clonogenic assays, flow cytometry, and programmed freezing and by including cryopreservation. Development was constant and seamless but four distinct periods are recognizable. First, guided and constantly encouraged by Professor E Donnall Thomas, was the use of an unfractonated mononuclear population derived from multiple sternal and iliac crest aspirations where complications, as in other centres, included rejection and, particularly troublesome, acute as well as chronic GVHD. The second was centred on CsA in association with Professor Jean Borel at Sandoz in Basle, leading to a decrease in the incidence and severity of the latter immunologic phenomena but not to their abrogation. Third was the opportunity of working with Professor Herman Waldmann and Dr Geoff Hale, first in Cambridge and latterly in Oxford, on immunosuppression achieved by ex vivo T-cell depletion within the broad ambit of the Campath users group. It was here that there was pioneered the alternative new approach of adding the anti-CD 52 MoAb only to the graft in what has become known as the in-the-bag technique. The fourth, securely based on early laboratory and clinical experiences, was a switch to the use of PBSCs mobilized into the circulation with stimulatory peptides. In 1995, this original transplant team relocated to a new academic centre in the private sector and has continued to actively refine the programme over the subsequent decade: the facility at Groote Schuur hospital continues independently. Early recognition that accountability for these expensive and high profile procedures was an important obligation led to consecutive transplants being reported to the International and Autologous registries and now continuing to the Centre for Bone Marrow Transplant Research in concert with the European Bone Marrow Transplant Registry. This disciplined approach has ensured that all data undergo constant audit and, on such a basis, underpin the unbroken accreditation extending over more than three and a half decades. With difficulties in finding sibling donors, a further achievement was the creation of The South African Bone Marrow Registry and now a proposal to also start a national transplant registry that will complement the survey currently being conducted, on a worldwide basis, by the European Group for Blood and Marrow Transplantation. It is concluded that a properly constituted and functioning multidisciplinary team can cost-effectively carry out immunohematopoietic stem cell grafting even in an under-resourced country with an outcome approximating that reported from recognized First World reference centres. The caveat is that, outside such comprehensive units, results may be less impressive, thereby arguing for resource allocation being directed to academically designated, rather than incentive-driven, preferred providers.


Keywords: immunohematopoietic SCT; introduction—development; South Africa

Professor E Donnall Thomas developed the concept and established the technical feasibility of BMT in a canine model. He and his team then courageously pioneered the translation of these orderly research studies into the clinic and first proposed what have become the currently accepted indications.1,2 From the start of these activities in Cooperstown to the Public Health Hospital in Seattle and finally to the Fred Hutchinson Cancer, he has through sheer talent inspired leadership and a unique compassion for patients and every member of his staff alike and attracted generations of Fellows, many of whom have gone on to sustain the ever clearer definition for the usage and unravelling of early and now late side effects. The hallmark of these graduates is his clear imprint of identifying a clinical problem and then increasingly applying cellular and

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molecular biologic techniques to understanding pathogenesis as a basis for refined or focused intervention. Small wonder that such achievements were recognized by the award of the Nobel Prize.

It would be inappropriate to describe the historical parallels that took place in South Africa without the preceding acknowledgement or an explanation of how the programme started in sub-Saharan. During the years as a haematology Fellow in Seattle with Professor Clement A Finch, there was regular contact and exposure to the excitement surrounding BMT. It would be both difficult—and invidious—to select from that group of dedicated investigators more than a representative mention of four who remain currently active, including Dr Rainer Storb, Dr Fred Appelbaum, Dr Joachim Deeg and Dr Joan Sanders with whom, among others, we are privileged to maintain an association that now extends back to more than 30 years.

One other event that cannot be overlooked is the momentous achievement of Professor Christian Neethling Barnard in carrying out the first human heart transplant in the world. It was into that receptive environment, prevailing at the University of Cape Town and Groote Schuur Hospital, that one Peter Jacobs had the great honour of being appointed as the Foundation Professor of Haematology. The inevitable question from Sir Richard Luyt and the appointments committee was what direction the new department had selected for particular focus! With no in-depth training in immunology and precious little in transplantation biology, it was with temerity that this emerging area of interest-BM grafting was chosen. And so the die was cast. The brief historical record that follows freely acknowledges unrestricted encouragement from innumerable colleagues of all persuasions, particularly during the early development with periods of frustration balanced by elation, and the many members of the multidisciplinary team who collectively made it a reality. It equally reminds us all never to lose sight of that extraordinary courage shown by patients and their families alike.

From individual centre and case studies to the meticulously orchestrated collection and analysis of data by the Centre for International Bone Marrow Transplant Research, indications in adults and children are now clearly defined (Table 1), whereas outcome remains the subject of continuous review and updating. Against this background, a number of relevant observations emerge that logically start with patient selection and a consideration of variables including age and comorbidity. It is here that the collective experience of the multidisciplinary team is crucial, with best results reflecting an under-appreciated centre effect.

All important is the conditioning of the recipient to accept the incoming graft, but, in selected instances and depending on diagnosis, it may be the need for further chemotherapy directed at eradicating residual disease. Historically, myeloablative preparation with high-dose chemotherapy or irradiation and more innovatively by the use of radioconjugates continues to undergo study. An area of particular interest has been the use of anti-CD 32 MoAb, which can be given either to a patient or a used ex vivo in what has become known as the in-the-bag technique.

Table 1 Traditional indications for transplantations

<table>
<thead>
<tr>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aplastic anaemia</td>
</tr>
<tr>
<td>Acute leukaemia</td>
</tr>
<tr>
<td>Myeloid</td>
</tr>
<tr>
<td>Lymphoid</td>
</tr>
<tr>
<td>Myeloproliferative diseases</td>
</tr>
<tr>
<td>Myelofibrosis</td>
</tr>
<tr>
<td>Myelodysplastic syndromes</td>
</tr>
<tr>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>Chronic leukaemia</td>
</tr>
<tr>
<td>Granulocytic</td>
</tr>
<tr>
<td>Lymphocytic</td>
</tr>
<tr>
<td>Lymphoma</td>
</tr>
<tr>
<td>Hodgkin</td>
</tr>
<tr>
<td>Indolent</td>
</tr>
<tr>
<td>Aggressive</td>
</tr>
</tbody>
</table>

Modified from: Forman S.J. The entry of patients only into clinical trials protocols is strongly favoured. Such structures provide for accountability and outcome analyses upon which rest accreditation. Also inclusive is the all-important quality-of-life evaluation and documentation of time-related side effects.

associated with this step has resulted in the description of reduced intensity regimens that make it possible to extend the upper age limit, but this may be offset by higher disease relapse rates. However, a reversal of the latter complication is possible in some cases by capitalizing on antitumour effects mediated by alloreactive T-cells in the form of delayed donor lymphocyte infusion. At this point remains the unresolved and challenging issue of optimum post transplant immunosuppression ranging from traditional corticosteroids with CsA and MTX through a wide range of new and potentially more effective—perhaps even safer—options.

This sophisticated form of treatment is best carried out in a dedicated reverse isolation unit or protected environment, but debate rages on precisely what defines such a physical facility or plant. Arguably, of far greater importance is investment in a properly constituted cross-disciplinary management team with, at least in Africa, input from practitioners of alternative or complementary medicine. Also, there is a need for a reliable and safe supply of blood and related products, a laboratory capable and registered as competent to carry out apheresis procedures, experienced in programmed freezing and licensed for cryopreservation in terms of locally promulgated acts for dealing with human tissues. Donor availability, particularly with increasing use of matched unrelated donors, and alternative sources including cord blood are those unique considerations securing access to tissue typing with immunogenetics accreditation. Transplants not carried out in such an organized, audited and accredited centre, designated as competent for both harvesting and grafting, are viewed as inappropriate and to be discouraged.

Then, in the short term defined as the first 100 days, is transplant-related mortality, which has a number of
Table 2  The annual post transplant evaluation

<table>
<thead>
<tr>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete blood count with differential</td>
</tr>
<tr>
<td>Hepatic and renal biochemistry</td>
</tr>
<tr>
<td>Viral screens for hepatitis and CMV</td>
</tr>
<tr>
<td>Endocrine evaluation: thyroid, sex hormones</td>
</tr>
<tr>
<td>BM studies as appropriate</td>
</tr>
<tr>
<td>Skin biopsy, if indicated</td>
</tr>
<tr>
<td>Pulmonary function tests</td>
</tr>
<tr>
<td>Ophthalmology consultation</td>
</tr>
<tr>
<td>Bone density documentation</td>
</tr>
<tr>
<td>Chest radiology</td>
</tr>
<tr>
<td>Eye and dental examination</td>
</tr>
<tr>
<td>Gynaecological exam/mammogram (women)</td>
</tr>
<tr>
<td>PSA (men)</td>
</tr>
</tbody>
</table>

Modified from: Lee S.14

In the interim, all individuals are seen as frequently as needed, but as a generalization, when asymptomatic, every 3 months. Once a year, more detailed assessment is prudent.

Table 3  Demography of South Africa

<table>
<thead>
<tr>
<th>Province</th>
<th>Population estimate as at 2007</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Western Cape</td>
<td>4,839,800</td>
</tr>
<tr>
<td>2. Northern Cape</td>
<td>1,102,200</td>
</tr>
<tr>
<td>3. Eastern Cape</td>
<td>6,966,200</td>
</tr>
<tr>
<td>4. KwaZulu-Natal</td>
<td>10,014,900</td>
</tr>
<tr>
<td>5. Free State</td>
<td>2,965,600</td>
</tr>
<tr>
<td>6. North West</td>
<td>3,394,200</td>
</tr>
<tr>
<td>7. Gauteng</td>
<td>9,638,100</td>
</tr>
<tr>
<td>8. Mpumalanga</td>
<td>3,336,200</td>
</tr>
<tr>
<td>9. Limpopo</td>
<td>5,402,200</td>
</tr>
</tbody>
</table>


Figure 1  Provinces of South Africa.

Standard of care using international norms. Arguments that such criteria do not apply to developing countries, where less good outcomes might be tolerated, are clearly specious as well as inappropriate! Such an attitude should not be entertained—and much less supported—particularly as there is evidence that precisely the opposite is true.16,17

It is against these observations that, first, the introduction and then the continuous development of immunohematopoietic SCT in South Africa over a 35-year period can now be detailed. There emerges the conclusion that at the present time they continue to provide life-saving and cost-effective, as well as resource-appropriate, therapeutic options in selected, properly managed centres that enjoy international designation.

Demographic considerations and current transplant activity

South Africa has a surface area of 1,219,080 km² (Table 3, Figure 1) with a population of 47,849,800 and a population density of 135/km². Complete records are available from the time BMT was started by the originally designated team (Table 4). Now, with the recent proposal that a national database be started, matching activities throughout the
Table 4 Transplant activities in South Africa

<table>
<thead>
<tr>
<th>Location</th>
<th>Principal investigator and reporting coordinator</th>
<th>First transplant date</th>
<th>Total</th>
<th>Allo</th>
<th>Auto</th>
<th>MUD*</th>
<th>Children*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cape Town</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>University-I: 1970–1994</td>
<td>Peter Jacobs and Lucille Wood</td>
<td>1974</td>
<td>179</td>
<td>147</td>
<td>32</td>
<td>0</td>
<td>Unknown</td>
</tr>
<tr>
<td>University-II: 1995 to date</td>
<td>Nicolas Novitzky</td>
<td>Data not provided 1996</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constantiaberg, Medi-Clinic</td>
<td>Peter Jacobs and Lucille Wood</td>
<td>1995</td>
<td>467</td>
<td>289</td>
<td>178</td>
<td>79</td>
<td>60</td>
</tr>
<tr>
<td>1995 to date*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Johannesburg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>University of Witwatersrand</td>
<td>Paul Ruff and Mary Farrel</td>
<td>2004</td>
<td>72</td>
<td>14</td>
<td>58</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Donal Gordon Private*</td>
<td>Paul Ruff and Mary Farrel</td>
<td>2000</td>
<td>86</td>
<td>38</td>
<td>48</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Medical Centre</td>
<td>David Brittain and Darryl Smith</td>
<td>2000</td>
<td>73</td>
<td>32</td>
<td>41</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Flora Private Clinic</td>
<td>Gart McMichael and Gaz Mac Beth</td>
<td>1999</td>
<td>33</td>
<td>4</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Garden City Private Clinic</td>
<td>Paul du Toit and Cecilia Botha</td>
<td>2007</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Park Clinic Private</td>
<td>Bernardo Rapoport and Phillie Merthelo</td>
<td>1998</td>
<td>70</td>
<td>0</td>
<td>70</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bloomfontein</td>
<td>Vernon Louw and Hymne Louw</td>
<td>Not yet activated</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Academic Hospital</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Durban</td>
<td>Vinod Jogessar and Natasha Sewpersad</td>
<td>2007</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pretoria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faerie Glen Private Hospital</td>
<td>Jackie Thompson and Hanseille</td>
<td>2006</td>
<td>44</td>
<td>12</td>
<td>32</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>University of Pretoria</td>
<td>Lydia Drosti and Jose Phatoli</td>
<td>2007</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mary Potter Private Oncology</td>
<td>Comrad Slabber</td>
<td>1996</td>
<td>232</td>
<td>35</td>
<td>184</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Centre*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ABDR = HLA A, B, DR; MUD = matched unrelated donor.
Peter Jacobs, Lucille Wood and JMG du Toit, Stellenbosch University—Tygerberg Academic Hospital.
Adam Noworthy, Devon Moodley and Georgis Demetriou—Joint Witwatersrand University and Donald Gordon Medical Centre.
Graham Cohen, Lydia Drosti, Ananda Vorster. Receipt of these figures from each participating centre is acknowledged with thanks to the Principal Investigator and reporting coordinator.

country can be collected hopefully through the existing Bone Marrow Registry.18 Such voluntary participation should, in future, make these activities a matter of general record. At the same time, South Africa has been included in a survey by a questionnaire circulated by the European Bone Marrow Transplant Registry gathering essentially the same figures. Clearly, these two activities need to be complementary.19 Logically, this exercise is but an initiating step that can easily be extended to formal outcome analysis. Implicit in such a rather more constructive endeavour will be the need to comply with ethics and research rules, including attention to issues that include donor confidentiality. This should be easy given the simple expedient of completing only already available, and circulated, relatively standardized data capture sheets!

Establishment of the Cape Town infrastructure

When this programme was inaugurated in 1970, blood was provided in glass bottles and platelets available only as single unit concentrates. To ensure that adequate transfusion support would be available, an IBM 2990 was secured by donation and this prompt action initiated a long and ongoing collaboration with Professor Jean Porter Hester.20 Although initially used for granulocyte transfusion, the apheresis technology rapidly became the anchor, together with the creation of a dedicated volunteer panel for donors, as one of the crucial moves towards realizing clinical transplantation. Physical facilities were limited and the start-up procedures were carried out with literally unlimited encouragement in the newly built block for cardiac transplantation through the courtesy of Professor Christian Neethling Barnard. As a result of his programme, histocompatibility testing also became available having only recently been introduced into our country. It was therefore possible to get class I by serology and some idea of class II by mixed lymphocyte reaction. These shortcomings were appreciated relatively soon and, in close collaboration with Professor Ernette du Toit, high-resolution typing became available with eventual immunogenetic testing approved in her laboratory.14 The activity of the South African Bone Marrow Registry is reflected in the latest statistics report (Table 5). The technical procedures of marrow processing, including characterization of the recovered mononuclear product, necessitated the development of a rabbit model in association with Professor Bruno Speck and Professor Aaloi Gratwohl in Basle to refine the methods, and the almost immediate establishment of flow cytometry and clonogenic assays using these transplants between strains showed that acute and chronic GVHD21 closely paralleled what was
being seen in the clinical programme. These manifestations were used to document the effects of G6A just made available by Professor Jean Borel at Sandoz in Switzerland. The same approach proved crucial in evaluating the administration of the Campath series of MoAbs to the recipient but subsequently showing that even more effective immunosuppression was possible by exposing the graft to the protein ex vivo in what has subsequently been described as the in-the-bag technique. This method has emerged as surprisingly rugged in abrogating classical acute GVHD and so substantially diminishing, almost to the point of extinction, the subsequently occurring chronic variant.

The clinical programme

The constant development, in a structured and actively evolving series, of experimental and clinical studies fell into four convenient but artificially distinct periods that characterize these activities in sub-Saharan Africa and particularly as chronicled at the University of Cape Town and Groote Schuur Hospital.

Unfractionated BM era mirrored experience occurring elsewhere in the world. Engraftment was often successful but plagued by graft failure and substantial morbidity as well as significant mortality consequent upon the syndrome of acute GVHD. Here, an often-profound injury to the skin, biliary endothelium and enterocytes, compounded by severe infection, responded only partially to immunosuppression with corticosteroids and MTX with extension to a severe sclerosing chronic form that created intractable disability in adults and children alike. In addition to the severe disability caused to recipients with devastating disorganization of family life, there emerged a more profound and often underplayed psychological injury to medical and nursing staff alike. This caused high staff turnover and understandable criticism from detractors of transplantation in general and more particularly to the use in hematology with the focus on the perceived worse side effects in children.

Cyclosporin A era started when this undecapeptide was isolated by Jean Borel in Sandoz and with our involvement in some of the earliest investigations of this biological breakthrough that was again thoroughly explored in the rabbit. Here, as also when the experimental studies were translated into the clinic, the incidence and severity, particularly of acute GVHD, was decreased but the broad range of complications changed surprisingly little. Nevertheless, this was a seminal step in being able to reverse the weakening confidence of the local medical community in the role of immunohematopoietic SCT and to set the stage for the continued active exploration of new and more effective agents.

Monoclonal antibody or campath era was in many ways the single biggest advance with which we were directly involved. Thus, the opportunity was afforded us of working with Professor Herman Waldmann and Dr Geoff Hale, initially in Cambridge and subsequently in Oxford, through participation in the scientific deliberations of the Campath users group. There was the innovative approach of employing selectively synthesized series of these Igs for

Figure 2. Overall survival in children. Kaplan-Meier analysis shows that at 6.8 years there is a stable plateau consistent with cure. It is notable that results are similar for idiopathic aplasia, Fanconi anemia and AML with nonsignificant differences for lymphoblastic leukemia and the remaining cases. There is also no difference in outcome for graft source but a slight benefit for the female gender, which did not attain statistical significance.

Figure 3. Overall survival in adults. One of the more interesting aspects of this programme is the similarity in survival by Kaplan-Meier analysis between consecutive patients with BM exposed to Campath 1-G-in-the-bag, peripheral blood treated with the same Ig or humanized variant and autografts. This particular approach is notable for the lower level of GVHD, and high remission rates in AML, but with an increasing incidence of CMV positivity that is the subject of ongoing investigation.

the alternative approach of effecting immunosuppression by means of relatively selective T-cell depletion. Experience in the animal transplant model, initially with the lytic IgM protein, showed GVHD to be beneficially affected and this could be duplicated in human allograft recipients with improvement in outcome. These events were enhanced first by the use of the opsonic IgG and then, more recently, by the humanized chimeric protein, confirmed in other countries.
Table 5  South African Bone Marrow Registry quarterly statistics

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of registered donors:</td>
<td>62 175</td>
<td>117*</td>
<td>62 223</td>
</tr>
<tr>
<td>HLA-AB typed</td>
<td>58 470</td>
<td></td>
<td>58 518</td>
</tr>
<tr>
<td>HLA-ABDR typed</td>
<td>3706</td>
<td></td>
<td>3775</td>
</tr>
<tr>
<td>% HLA-ABDR typed</td>
<td>5.9%</td>
<td></td>
<td>6.0%</td>
</tr>
<tr>
<td>Searches for Donors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preliminary searches:</td>
<td>2432</td>
<td>70</td>
<td>2502</td>
</tr>
<tr>
<td>Local Haematologists</td>
<td>490</td>
<td>20</td>
<td>510</td>
</tr>
<tr>
<td>International Registries</td>
<td>1942</td>
<td>50</td>
<td>1992</td>
</tr>
<tr>
<td>Activated searches South African patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New activated searches this quarter</td>
<td></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>All active searches this quarter</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>South African patients receiving matched unrelated transplants:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local donor</td>
<td>109</td>
<td>4</td>
<td>113</td>
</tr>
<tr>
<td>International donor</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>% Local donor</td>
<td>79</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td>PBSC donations by South African donors:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local recipients</td>
<td>37</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>International recipients</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>% Local donor</td>
<td>27.5%</td>
<td>26.5%</td>
<td></td>
</tr>
</tbody>
</table>

*Net gain for the period but who have not yet been transplanted. Note: 494 deletions this quarter.
*All open patient files, including patients for whom a donor has been found.
*First MUD transplants with thanks to Professor Ernette Du Toit, Mrs Terry Schlapoff and Mrs Veronica Borrill for permission to use these data.

Table 6  Cost estimate for stem cell grafting in South Africa

<table>
<thead>
<tr>
<th>Description</th>
<th>Rand</th>
<th>Dollar</th>
<th>Euro</th>
<th>Pound</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBSC collection</td>
<td>25 000</td>
<td>3576.89</td>
<td>2592.00</td>
<td>1702.39</td>
</tr>
<tr>
<td>Physician’s fees—daily management</td>
<td>60 000</td>
<td>8104.53</td>
<td>6019.20</td>
<td>4085.75</td>
</tr>
<tr>
<td>Ward fees</td>
<td>80 000</td>
<td>10806.00</td>
<td>8025.60</td>
<td>5447.66</td>
</tr>
<tr>
<td>Chemotherapy conditioning</td>
<td>40 000</td>
<td>5403.02</td>
<td>4012.80</td>
<td>2723.83</td>
</tr>
<tr>
<td>Projected antibiotic usage</td>
<td>20 000</td>
<td>2701.51</td>
<td>2066.40</td>
<td>1361.92</td>
</tr>
<tr>
<td>Other pharmaceuticals</td>
<td>6500</td>
<td>877.99</td>
<td>652.08</td>
<td>442.62</td>
</tr>
<tr>
<td>Consumables</td>
<td>12 000</td>
<td>1620.91</td>
<td>1203.84</td>
<td>817.15</td>
</tr>
<tr>
<td>Radiotherapy conditioning, etc</td>
<td>55 279.40</td>
<td>7466.89</td>
<td>5545.63</td>
<td>3764.29</td>
</tr>
<tr>
<td>Placement of central venous catheter</td>
<td>6 500</td>
<td>877.99</td>
<td>652.08</td>
<td>442.62</td>
</tr>
<tr>
<td>Bone marrow aspiration</td>
<td>5000</td>
<td>675.37</td>
<td>501.50</td>
<td>340.48</td>
</tr>
<tr>
<td>Blood and related products</td>
<td>50 000</td>
<td>6753.77</td>
<td>5016.00</td>
<td>3404.79</td>
</tr>
<tr>
<td>Pathologist’s fees</td>
<td>20 000</td>
<td>2701.51</td>
<td>2006.40</td>
<td>1361.92</td>
</tr>
<tr>
<td>Radiologist’s fees</td>
<td>15 000</td>
<td>2026.13</td>
<td>1504.80</td>
<td>1021.44</td>
</tr>
<tr>
<td>Total</td>
<td>408 779.40</td>
<td>55 216.03</td>
<td>41 008.75</td>
<td>27836.16</td>
</tr>
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</table>

At about the midpoint of these collaborative studies, innovative experimental evidence in our laboratory demonstrated that the addition of Ig to the graft, before infusion alone and without any subsequent immunosuppression using antibody or drugs, impacted favourably on acute GVHD. This syndrome virtually disappeared, although a late-presenting mild acute variant was recognized limited to the skin, being grade 1 and typically responding to topical steroids but seldom needing systemic administration. This was the birth of the in vitro, ex vivo or, as it has subsequently become known, Campath in-the-bag technique.10

Overlapping with these developments was the recognition that a stem cell population having many characteristics similar to those derived from the BM could be obtained by the much simpler expedient of recovery from the peripheral circulation usingpheresis technology, with enhancement resulting from the donor receiving stimulatory peptides in the form of G-CSF.28 A new observation emerged in that CMV infections, which had not been particularly prominent while using marrow, now required increased constant monitoring and prompt administration of gancyclovir to avoid progression from viraemia to the much more hazardous situation of clinical disease typically in the lung or the gastrointestinal tract.

The current era, spanning the last 12 years, has been particularly illuminating in the context of an under-resourced area where state support for teaching hospitals continues to relentlessly erode so that commercial ventures have become more receptive to accepting tertiary-level
responsibilities. Thus, it has been possible to relocate, within a single Facility, most of the original team to a privately based Academic Department of Hematology and Bone Marrow Transplant Unit that incorporates the Searl Research Laboratory for Cellular and Molecular Biology. The experiment has been successful, proving that it is realistic to maintain the consecutive patient reporting system first to the International and then to the Autologous registries that are currently combined into the Centre for Bone Marrow Transplant Research. This period demonstrates the feasibility of meeting the audit criteria for continued accreditation as both a transplant and harvest centre now extending for an unbroken period in excess of three decades, as well as accommodating the need for matched unrelated volunteer donors. The latter sparked the need to start a donor registry that has become a national entity providing tissue-typing services for other centres in the country, regularly interacting with corresponding facilities and participating in searches from elsewhere in the world with activity recently updated. The standard is such that endorsement is maintained from the American National Donor Programme as well as the European Bone Marrow Transplant Registry. In parallel outcome has been analysed and reported in both children and adults (Figure 2) and adults (Figure 3), whereas the particular relevance of these procedures in patients with lymphoma continues to attract our ongoing and special attention.

An important consideration in the Third World is to balance the need for these high cost procedures against resources available in state hospitals having a limited budget and a competing private sector where restraints are of a slightly different nature dictated by the particular insurance plan available through managed care. In order that some idea of national activity can be gauged, it was recently proposed that a BM transplant registry be established within the ambit of the existing national body, the South African Bone Marrow Registry. The argument in favour of such a base is that it would allow comprehensive or inclusive recording of autologous and all forms of allogeneic transplantation and include alternative sources, such as cord blood and matched unrelated volunteer donors, and so in a non-partisan way. It would also permit the government, through the Department of Health, access to reliable statistics as to activity of the teams, whether they be in university hospitals or private sectors. Additionally, it would anticipate future needs for appropriate regulation that may be promulgated in terms of the Human Tissues Act. Here, particular issues relate to harvesting, any form of processing or manipulation of grafts and subsequent cryopreservation while monitoring the movement of these human products not only within the country but also as a cross-border between countries through the medium of collaborating international registries.

Conclusion

The basis for this national experience can be factually recorded in three phases of past achievement and present status with a projection for the future. Historically, the experimental hematology and subsequent systemic transplantation into the clinical programme documents the capability of an under-resourced country to provide advanced and life-saving interventions as dictated only by the genuine needs within the community, across the age spectrum and catered for by the state or private sector, even where costs are a major consideration (Tables 5 and 6). It is equally clearly demonstrated that in those few facilities that have elected to meet the criteria for ongoing audit and accreditation from international peers, designation is possible as a donor and harvest centre leading to participation in a worldwide community linked through registries that share search and provision of matched unrelated volunteers when these are not available locally. Finally, it is time to survey the activity within our borders and to this end the reporting of consecutive procedures by each practice has proposed forming a registry to operate within the ambit of the nationally constituted South African Bone Marrow Registry. The latter initiative is given sharp focus by a similar approach already launched by the European Bone Marrow Transplant Registry, and both should be mutually complementary. Such a proactive move would have the capacity for expansion to document outcome, although this would require appropriate ethics and research monitoring. A further attraction is the creation of a reliable database providing comparisons between different regions of the world and, in the local context, a basis to guide and educate practices in terms of regulation for transfusion practices and handling human tissues. The natural end point becomes academic recognition for properly accredited programmes to safeguard future scientific and training requirements while simultaneously reducing any need for commercial or incentive-driven providers.

Conflict of interest

Neither author declared any financial interests.

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CHAPTER 2

DEVELOPMENT OF NEW MODALITIES

FROM 1980
The experimental model

The rabbit was chosen although much of the ongoing work, worldwide, at that time was murine. The management of animals was in a vivarium and under constant surveillance by the relevant University research committee with attention to every detail of proper experimentation techniques and in accordance with all prevailing recommendations. This carefully considered decision was based on an opportunity to collaborate with colleagues in Basle – Bruno Speck and Alois Gratwohl. They had developed two distinct inbred strains and characterised these immunologically as being suitable to examine not only engraftment but also rejection and acute as well as chronic graft-versus-host disease. To constantly evaluate the results outcome was regularly presented at international meetings. These animals were also of a suitable size and could be handled safely during the cardinal first steps of marrow recovery in addition to providing adequate material for the processing, using existing morphologic criteria, with available mononuclear separation techniques. Techniques were introduced for repeated blood sampling in small animals and bone marrow reconstitution using a block grafting technique explored. After feasibility had been established preliminary studies evaluated immunosuppressive therapy with Corynebacterium parvum as modulating graft-versus-host disease in allografted pairs.

The specialised transplantation laboratory

Experience in manipulation of different cell populations required new collection and separation methods, accumulating experience in microbiology and safe operation of laminar - down flow environments for ex vivo cultures. Initially there was characterisation of progenitors and stroma focussing on patterns of growth determined by both physiological cytokines and alterations resulting from additional stimulatory peptides. Such reference or baseline ratios defining colonies and clusters each with distinctive cytomorphology. A modification was needed to record, with appropriate staining and innovative fixation, a means to retain the pattern. These became part of permanent reference library for the composition and behaviour of quite different populations found in the marrow of both the animals and human samples.
Additionally cytogenetics, flow cytometry and cytochemical techniques were introduced, instruments calibrated, normal ranges defined and these innovations extended to characterising fluid phase tumours\textsuperscript{72-74}.

\textbf{Creation of essential infrastructure in the clinic}

As these preparative stages were being completed the role of this procedure attracted increasing medical\textsuperscript{75} and nursing attention\textsuperscript{76}. Among these were the technicalities for the adequate sampling of bone marrow\textsuperscript{77,78} and the parallel interaction with other local scientists exploring such anticipated problems as chemotherapy-induced cardiomyopathy\textsuperscript{79,80}.

Substantial new challenges were predicated by lack of modern transfusion medicine exemplified by the fact that whole blood was supplied in bottles and platelets as single units\textsuperscript{88,89} leading to introduction of new methodology\textsuperscript{91}. These obsolete two practices were incriminated in increasing the risk of antigenic exposure and posed insurmountable barriers for any projected bone marrow transplantation program.

It was therefore necessary to establish an apheresis or cell support unit\textsuperscript{78} that brought with it new problems. Also the dependence on patients for repeated venous access and, yet again experience with the rabbit model pioneered the forerunner of the Hickman line\textsuperscript{82}. The next logical step was to obtain a discontinuous and then the first continuous blood flow cell separator on the continent which was commissioned and after all the operating characteristics carefully documented progress to setting up an appropriate component support program\textsuperscript{83-85}. This was followed by establishing a dedicated in-hospital platelet volunteer program\textsuperscript{86,87} and use of this methodology for treating sickle crisis\textsuperscript{88}. After a probationary period consistent incident-free outcome led to the two investigators being licensed for all these procedures on authorisation from the Department of Health.
On the basis of such experience operating procedures were developed for the recovery of granulocytes\textsuperscript{89} followed by the all important and cardinal studies in collection and with subsequent cryopreservation of human stem and progenitor cells as the anchor for bone marrow transplantation\textsuperscript{90}. Neither was the potential side-effects of the technique overlooked\textsuperscript{91} and in a very forward-looking and visionary way donor safety\textsuperscript{92} introduced and continues to this day\textsuperscript{93,94} although the focus is shifting to longer term observation in the light of recent reports\textsuperscript{95,96}.

\textit{The scene is finally set}

Securely founded on these years of systematically developing, in a structured and stepwise fashion, the standardised laboratory methodology moved through translational research taking each innovation into the clinic. All the technology supported by allocation of the necessary resources in terms of inpatient and outpatient beds, medical nursing and other paramedical staff led to the first successful bone marrow transplantation in this country\textsuperscript{93}. 
THE EFFECT OF CYCLOSPORIN A ON THE SURVIVAL OF RABBITS FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION. P. Jacobs, D. Barends, J. Parker, G. Manuel, The Department of Haematology, The University of Cape Town and Groote Schuur Hospital, Observatory, Cape - South Africa; University of the Western Cape; Provincial Blood Grouping Laboratory.

Graft-versus-host disease (GVHD) is the most important cause of death in patients and animals following allogeneic bone marrow transplantation. Neither attempts to prevent the syndrome with methotrexate or treatment of the established disease with corticosteroids or antithymocyte globulin have significantly altered morbidity or mortality. The immunosuppressive agent cyclosporin A (CSA) is reported to be effective in therapy of GVHD in rodents. The present study was undertaken to extend this observation. Using a previously described animal model New Zealand White strain of rabbits were irradiated with 1,200 rads mid-plane to render them aplastic; this was followed by infusion of a bone marrow suspension obtained from R strain rabbit donors. The recipients were randomly allocated to receive either no treatment or 28 consecutive daily intramuscular injections of CSA (10 mg/kg). There was no evidence of renal, hepatic or haematopoietic protractility associated with the CSA administration. Survival figures at 28 days in the control animals were 1/3 (12.5%) and in those receiving CSA this was 11/18 (61%): at 100 days there were no survivors in the untreated animals and 6/18 (36%) of those receiving CSA were alive and well. Histology of tissue obtained at autopsy from the controls showed changes compatible with GVHD in all the animals examined. In the CSA treated animals dying before day 28 there was a lower incidence of these findings and death was due to infection. However, after 28 days GVHD was again manifest affecting predominately the gut. In none of our animals was there evidence of malignancy developing in the long-term survivors. It is concluded that, as in rodents, the administration of CSA to rabbits undergoing allogeneic bone marrow transplantation significantly prolonged survival.

This study was supported by grants from the Medical Research Council, The National Cancer Association and the Harry Crossley Foundation of the University of Cape Town. We thank SANRP for a donation of cyclosporin A.
Jacobs P, Bareford D, Parker JR, Manuel G.
The use of Cyclosporin A to increase the duration of survival of rabbits following allogeneic bone marrow transplantation.
AN EVALUATION OF CYCLOSPORIN A IN REDUCING THE INCIDENCE OF GRAFT VERSUS HOST DISEASE IN RABBITS UNDERGOING ALLOGENIC BONE MARROW TRANSPLANTATION.

by P. Jacobs, D. Bareford, J.R. Parker, B. Milns & G. Manuel, University of Cape Town Leukaemia Centre & Department of Haematology, Groote Schuur Hospital, Observatory, Cape Town, South Africa

A PRELIMINARY REPORT

INTRODUCTION

Bone marrow transplantation using HLA-identical and MLC non-reactive siblings is the accepted form of treatment for patients with severe acute aplastic anaemia and immunodeficiency disease, and it has given encouraging early results in leukaemic patients when transplanted in their first complete remission.

In a recent study of 30 patients with severe acute aplastic anaemia who had been minimally exposed to antigens contained in blood the projected survival at six years is 75% (1). In such selected individuals the incidence of graft rejection, previously in the range of 25% - 60%, is greatly reduced. Nevertheless, graft-versus-host disease still affects over half the patients and causes long-term problems in 20% of the survivors.

In leukaemic patients undergoing bone marrow transplantation in remission,
survival figures are over 60 %, and rejection is a lesser problem (2,3). These results should be compared to long-term survival with conventional cytotoxic chemotherapy, which is less than 10 %. Not surprisingly, there is current enthusiasm for bone marrow transplantation in these patients wherever an HLA-identical and MLC non-reactive sibling is available but graft-versus-host disease occurs with a similar frequency to that found in patients with severe acute aplastic anaemia who have been transplanted.

In general terms, this process is the major cause of morbidity and mortality in transplanted patients and the incidence appears to increase if poorly matched siblings are used as donors. The pathophysiology is the infusion and survival of immunocompetent cells contained in the bone marrow graft into immunologically incompetent recipients. The exact cellular mechanisms mediating the clinical syndrome are not fully understood. It is thought that the antigens on the cells of the recipient, probably determined by the major histocompatibility locus, are recognised as foreign by the engrafted immunocompetent cells and these are attacked with resulting cell death consequent upon the formation of T-killer cells.

EXPERIMENTAL STUDIES

An animal model has been developed to study the effect of a number of agents on the incidence of graft-versus-host disease. Initially, studies were directed at the effect of parenteral Corynebacterium Parvum and subsequently to assess the effect of the administration of Cyclosporin A on the severity of the process and also on animal survival. Cyclosporin A is an anti-lymphocyte peptide derived from two species of fungi (Cyclindrocarpon lucidum booth and Thickodermia polysporum) having the capacity to suppress cell-mediated immunity in experimental skin graft rejection, graft-versus-host disease, and experimental allergic encephalomyelitis in rodent models. It has also been successfully used to prolong allograft survival in rabbit and dog kidney, and pig and rat heart transplantation studies. More recently Cyclosporin A has been evaluated in human renal and bone marrow transplantation, and early results are encouraging.

Studies reported from the Royal Marsden Hospital in England on the use of Cyclosporin A for treatment of graft-versus-host disease in humans, showed that the skin manifestations abated in two days but no improvement in liver function was noted, and four of the five patients died of liver failure (4). In a subsequent trial using Cyclosporin A for prophylaxis, 21 patients were given the agent starting immediately before transplantation in a dose of 12.5 mg/kg for up to six months (5). There appears to be an appreciable reduction in the incidence of graft-versus-host disease, and five of those patients have now been off the drug for up to three months and continue to do well.

Side-effects include renal, hepatic, and gastrointestinal toxicity. Rising blood urea and creatinine have been observed in most patients, but this is reversible and can be controlled by adjustment in Cyclosporin A dosage: Approximately 50 % of the bone marrow transplant patients show a rising bilirubin and this may reach levels of 10 mg %. Additionally, hepatic enzyme elevation may occur. These abnormalities in liver function tests appear to be reversible. A number of patients show anorexia with conse-
quent weight loss but these symptoms occur predominantly with oral drug administration.

EXPERIMENTAL STUDIES CARRIED OUT AT THE UNIVERSITY OF CAPE TOWN LEUKAEMIA CENTRE.

A rabbit model was used in which R-strain females donated bone marrow to New Zealand White male recipients. The latter were individually housed and received prophylactic antibiotics for four weeks following transplantation. Twenty-four hours before the procedure, the recipients were rendered aplastic with 1.200 rads total body irradiation delivered from a cobalt point source. A mononuclear suspension of donor bone marrow cells was infused intravenously into the irradiated recipients and the dose standardised between 2.0 and 4.0 x 10^8 nucleated cells per kg. The recipients were randomly allocated to three groups. Group 1 were radiation control animals which received no marrow cells. Group 2 were transplantation or graft-versus-host disease controls; these received no modifying drugs before or after transplantation. Group 3 were the Cyclosporin A study group and received 10 mg/kg parenterally between the first and the 28th post-transplantation day.

RESULTS

All radiation control animals died on or before day 7. A small number of rabbits in both the transplantation control and the Cyclosporin A group died before day 7 due to infection or haemorrhage before engraftment had occurred and peripheral granulocytes and platelets had reached normal levels. These animals were excluded from the study since the design was to investigate the effect of Cyclosporin A on the incidence of graft-versus-host disease, and the latter would be more likely to occur once the donor marrow had become established in the recipient.

Of the animals suitable for analysis 10% in the allograft control series (group 2) were alive at day 40, and none at day 100. In the Cyclosporin A group the corresponding figures were 44% and 33%, respectively.

Routine haematologic assessment confirmed that engraftment had occurred at the same time in both groups: usually between days 5 and 8. There was no difference in peripheral haemoglobin, platelet, or white cell count for the period of observation (100 days).

Postmortem examination of the transplant (graft-versus-host disease) control group showed an appreciable number (66%) dying with histological manifestations of this syndrome. By way of contrast, only 25% of those in the Cyclosporin A treated group died with comparable organ histology. The remaining animals died of incidental causes such as perforated gastric ulcers, septicaemia, and either interstitial or broncho-pneumonia.

In none of the animals was evidence of lymphoma demonstrated.

It is notable that in this pilot study the Cyclosporin A was discontinued at day 28 and further studies are needed to determine whether longer periods of administration will be associated with further improvement in survival data and additional reduction in the histologic evidence of graft-versus-host disease.

In a separate study on normal rabbits given Cyclosporin A in a matching dose for 28 days no rise in hepatic
enzymes or renal function was demonstrated.

CONCLUSION

This study in experimental animals is encouraging in demonstrating a clear reduction in histologically proven graft-versus-host disease in the allo-transplanted rabbit model where animals receive prophylactic Cyclosporin A.

PROJECTED STUDIES

Studies are in progress to examine Cyclosporin A toxicity as a function of plasma level determined either on high pressure liquid chromatography or by radioimmuno-assay and also to define the effect of prolonged administration of Cyclosporin A on both the incidence of graft-versus-host disease and on survival in prophylactically treated animals. Studies are also being done to correlate these findings with additional, in vitro, measurements of lymphocyte and macrophage function and to examine the effects of Cyclosporin A administration on in vitro marrow culture and on the rate of immunologic reconstitution.

These studies are the basis for the ongoing clinical transplantation programme where patients with severe acute aplastic anaemia and those with leukaemia achieving their first complete remission are transplanted from suitably matched siblings and receive this agent as the only post-transplantation immunosuppressive therapy.

A simple method for repeated blood sampling in small animals

PETER JACOBS and LIEVE ADRIAENSSENS Seattle, Wash.

A method is described for introducing a shunt into the carotid artery of small animals. A subcutaneous reservoir remains easily accessible between the cannulas allowing frequent collections of relatively large volumes of unhemolyzed blood. Such sampling is convenient and can be accomplished with minimal discomfort to the animal, which is gently restrained but not anesthetized.

Many experimental situations in small animals necessitate repeated blood sampling. On occasions the specimens collected need to be of relatively large volume or it may be essential that they are free from hemolysis. On other occasions more complex manipulations such as exchange transfusions are required. In many situations some degree of success can be achieved by repeated cardiac puncture or the use of multiple venipunctures, but both of these techniques have well-recognized limitations. The following method is described because of its simplicity and the case with which repeated, nonhemolyzed samples may be collected over prolonged periods of time.

Material and methods

In adult rabbits or cats a modified heparin lock* (Fig. 1) is introduced subcutaneously so that the reservoir lies between the cannulas and the free end of the cannula is located in the left carotid artery. The right carotid artery may be used, but the anatomy of the

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No reprints will be available.
great vessels is such that the introduction of the cannula is a little more difficult and mechanical obstruction and thrombosis occur with greater frequency.

The animals are lightly anesthetized with halothane so the shunt can be inserted, and a one-centimeter skin incision is made in the midline between the carotid. The animal is then turned onto its back and a 2 to 3 centimeter skin incision is made in the neck from the level of the thyroid cartilage downward. The free end of the hopia-look is firmly grasped in a pair of curved forceps and directed subcutaneously into the back of the neck and then anteriorly to emerge at the top of the wound overlying the trachea (Fig. 2).

After the reservoir is adjusted subcutaneously, the forceps are withdrawn and the cannula is gently tensioned. The fat overlying the trachea is divided, and the left sternomastoid muscles are dislocated medially. This maneuver will expose the carotid artery and
Repeted blood sampling in small animals

Fig. 3.

the accompanying vagus nerve. The artery is ligated at the upper level of the thyroid cartilage, and the cannula is introduced 3 cm. into this vessel through a small nick made immediately below this ligature (Fig. 3). Once the cannula is in position, without any kinking, it is secured with a second ligature approximately 1 cm. below the first. The 2 wounds are closed with either clips or sutures.

While a completely sterile surgical technique might be employed, we have found this unnecessary and administer a 3 day course of penicillin and streptomycin to prevent post-operative infection. Blood is sampled by passing of a 20 gauge needle through the skin and directly into the heparin lock via its rubber cap; in this way any volume may be withdrawn as frequently as necessary. In order to keep the shunt patent, 1 ml. of saline containing between 50 and 100 units of heparin is injected after each sample collection and, when the shunt is not being regularly used, once every 24 hours. The volume of heparin should be adjusted to the frequency with which sampling takes place.

Discussion

Standard techniques for blood collection in small mammals include multiple cardiac puncture and the use of the marginal vein or central artery of the ear in the rabbit or from subcutaneous veins in animals such as small dogs and cats. In the former situation a small number of animals die regardless of the ability of the operator. The deaths are probably related to occasional trauma to the
coronary artery or disruption of valves or conducting tissue. On occasion significant hematothorax follows accidental division of such vessels as the internal mammary artery. While these situations are not serious in donor animals, they may become significant when death or unwanted blood loss follows manipulation during a course of a particular experiment. Similarly, collection of blood from the ear vessels cannot be repeated with any great frequency and often results in samples of inadequate volume or in the presence of significant degrees of hemolysis.

Approximately 50 shunts have been used in studies carried out in rabbits and cats, and these have functioned satisfactorily for periods as long as 8 weeks. The shunts described have, in our hands, had no operative mortality, and we have not recognized infection in the path of the shunt or within the heparin-lock itself.

Problems do arise in those circumstances where the rubber cork is inadvertently pulled out during sample collection. If such an accident is unrecognized, exsanguinating hemorrhage may occur. In situations where very frequent sampling takes place, some degree of leak inevitably occurs backward through the punctured rubber cork and may also give rise to hematoma formation with subsequent local infection; this may be avoided by changing the rubber cork every second or third day.

On rare occasions occlusion of the shunt has occurred. This has always followed inadequate care and, if recognized early enough, can be unblocked by injection of 2 ml of heparinized saline forcibly into the reservoir. Twice, this has resulted in convulsions and death of the animal due to cerebral emboli. This complication has not been seen when the cannulas are located in the left carotid artery. Care must be taken with these retrograde injections since rapid injection of cold saline, particularly down the right artery, can produce sudden cardiac arrest possibly due to cardiac arrhythmia.

The occasional use of both carotid arteries in a single animal has not produced any neurological disturbance, possibly because of a well-developed vertebro-basilar circulation. In other animals cannulas have been introduced simultaneously into the carotid artery and the jugular veins, and, although venous sampling has usually been adequate, more frequent occlusion occurs in the jugular vessels.

It is a pleasure to thank Dr. Clement A. Finch for helpful suggestions in preparation of this manuscript and for the use of facilities in his laboratory.
Bone Marrow Reconstitution Using a Block Grafting Technique

JOAN R. PARKER, SUSAN P. TAYLOR, G. MANUEL, P. JACOBS

SUMMARY

Experiments in rabbits with radiation-induced bone marrow aplasia have demonstrated that intramedullary block grafting is a feasible procedure. This technique requires surgical interference and offers no advantage over intravenous reconstitution. We therefore suggest that it has no value in therapeutic bone marrow transplantation.


The transplantation of bone marrow from HL-A identical and MLC non-reactive donors into patients with aplastic anaemia results in permanent recovery of marrow function in approximately 50% of cases. The currently accepted technique for such reconstitution is the intravenous infusion of a mononuclear suspension, and the use of this method in animals and man has been well documented. However, a number of cells are unavoidably damaged during preparation of the suspension, and there may thus be some selective loss of stem cells. Furthermore, a large number of the infused cells are sequestered in the lung following administration of the graft and, while this is likely to be transient, their exact fate remains unknown. These observations have suggested a need to explore alternative methods for reconstitution. It seems logical that if the marrow could be replaced directly into the bone marrow cavity in particle form, both of the above limitations would be avoided. In addition, it is theoretically possible that if the architecture is intact and cell relationships are therefore undisturbed, recovery may be significantly enhanced.

This study was undertaken to explore the feasibility of such an intramedullary grafting technique, using lethally irradiated rabbits as a model.

METHODS

Rabbits were selected both for easy manipulation and for the ready availability of bone marrow from the femora. The animals had an average body weight of 2 kg. A dose of 1 200 rads was delivered to the horizontal midplane from a cobalt source, and this invariably resulted in death of the control animal from bone marrow damage between 4 and 12 days after irradiation, without causing significant gastro-intestinal or other radiation-induced disturbance. It was important to deliver the radiation with the anaesthetised rabbit lying on its stomach, since in the lateral positions the femora were at different heights in the radiation field, and this resulted in an uneven distribution of damage to the marrow.

To determine the adequacy of a marrow graft from the femur in preventing death after lethal irradiation, one femur, or both, was shielded with lead during irradiation, and the recovery of peripheral blood values and bone marrow was measured.

The technique for removing the femoral marrow prior to intravenous infusion was then developed in the rabbit. A small hole was drilled at the level of the greater trochanter to admit a 20-gauge needle, and a slightly larger hole was drilled at the lower end of the bone into which a Teflon tube of 1.0 mm internal diameter fitted tightly. The contents of the femur were then washed out with tissue culture medium (TC 199, containing 20% autologous serum and 25 units of preservative-free heparin), by gentle pressure on a syringe containing the medium and attached to the 20-gauge needle. The marrow was converted into a mononuclear suspension by the method previously described. After exposing the rabbits to 1 200 rads, whole body irradiation, the autologous suspension was reintroduced into the marginal vein of the ear (group I). An aliquot of the marrow was retained for measurement of colony formation in soft agar, for determination of cell viability, and for differential counting.

The critical question of intramedullary reimplantation was then systematically explored. In initial experiments the femur was de-roofed and the marrow was curetted out (group II). After irradiation, the autologous marrow was repacked into the femoral cavity en bloc, and the excised bone fragment was replaced and secured with either internal mechanical fixation or acrylic cement.

In the third group of animals (group III), a larger hole (0.5 cm in diameter) was drilled at the lower end of the femur to avoid the de-roofing procedure. The marrow was removed by applying pressure with the syringe and a 20-gauge needle at the upper end of the femur, so that a solid marrow core was extruded intact. The animal then received lethal whole-body irradiation, and the block of marrow was reintroduced retrogradely into the now vacant femoral cavity via the lower hole. This procedure prevented the femoral fractures which occurred in group II.

In a further group of animals (group IV) the marrow was removed as described above, but was converted into coarse particles by passage through a screen of 0.6 mm
mesh size before retrograde intramedullary replacement, for which the same procedure as in group III was used.

RESULTS

The effect of the varying doses of radiation on survival is shown in Fig. 1. The L.D. was 1 200 rads, and this point was clearly defined. It is particularly significant that this dose level caused total and irreversible bone marrow aplasia without other complications from the irradiation. Specifically, haemorrhage of the gastro-intestinal tract or pulmonary changes were not demonstrable.

All 12 animals in the control group died from overwhelming infection within a period of 4-12 days. Postmortem examination showed that the organs were heavily infected with micro-organisms, which were prominent within the vascular system. None of the recognised short-term effects of irradiation were demonstrable at postmortem examination.

\[ \times \text{ALIVE} \quad \bullet \text{DEAD} \]

---

**Fig. 1. The relationship of radiation dosage to survival. The L.D. is 1 200 rads delivered to the horizontal midplane of the rabbit.**

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In the control animals haemoglobin levels of between 10 g/100 ml and 12 g/100 ml were recorded. The average platelet count was 12 000/μl but, significantly, bleeding was not a problem, and leucocyte counts varied between 500/μl and 1 000/μl, without any granulocytes.

In the animals where one or both femora had been shielded (Fig. 2), peripheral blood values all returned to normal. The rise was quicker where both femora had been shielded, although during the 30 days of observation the values never returned to completely normal levels.

Of the 8 animals that received the standard irradiation, followed immediately by intravenous infusion of autologous marrow (group I), 6 survived the procedure with permanent recovery of marrow function (Table I). The 2 rabbits in this group that died, did so from infection before engraftment had occurred.

---

**ALIGHT**

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A. CONTROL

B. TWO FEMORA SHIELDED

C. ONE FEMUR SHIELDED

---

**Fig. 2. The relationship between leucocyte count and post-irradiation interval. Irradiated animals (control group) survived 4-12 days without reconstitution. Shielding of both femora led to a more rapid return of the leucocyte count to normal than when one femur was shielded. After 30 days the leucocyte count had not reached the basal level.**

---

**TABLE I. COMPARISON OF SURVIVAL**

<table>
<thead>
<tr>
<th>Group</th>
<th>Method of reconstitution</th>
<th>Survivors</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Nil</td>
<td>0/12</td>
<td>0</td>
</tr>
<tr>
<td>Group I</td>
<td>Intravenous</td>
<td>6/8</td>
<td>75</td>
</tr>
<tr>
<td>Group II</td>
<td>Block</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>Group III</td>
<td>Large fragments</td>
<td>2/8</td>
<td>25</td>
</tr>
<tr>
<td>Group IV</td>
<td>Small fragments</td>
<td>3/6</td>
<td>50</td>
</tr>
</tbody>
</table>

Aliquots of the monocellular suspension which were studied in the laboratory revealed a viability of intact cells in excess of 95%, and bone marrow differential cell counts were in close agreement with those previously reported. Colony formation in soft agar was used to predict the likelihood of engraftment, but, apart from a broad correlation with nucleated cell counts, was found to have limited value when used in this context.

None of the 4 animals in group II, in which the femur had been de-roofed, survived for more than 12 days. These animals died from overwhelming infection, and postmortem examination showed extensive necrosis of the replaced marrow.

Of the 8 animals in group III, in which the marrow was replaced en bloc into the femur by the retrograde route using a syringe, 2 survived. In these animals normal bone marrow function was demonstrated, but large areas of marrow necrosis were evident in biopsy specimens. Among the animals that died, the striking feature was infection, with a great deal of phagocytic activity in which macrophages were ingesting the necrotic bone marrow.

In group IV, 3 of the 6 rabbits recovered, with adequate bone marrow function. Fig. 3 shows the recovery of the leucocyte count in groups I and IV. Significantly, the rate of increase was no faster after intramedullary than after
intravenous reconstitution, and the animals in both these groups showed a more rapid haematological recovery than those in the group in which one or both of the femora had been shielded.

![Graph](image)

Fig. 3. Comparison of the leucocyte count in intravenously grafted animals (group I) and in those receiving intra- medullary grafts of small particles (group IV). Note that the rate of recovery is not significantly different.

**DISCUSSION**

This radiation model of bone marrow aplasia has been found practical because LD<sub>50</sub> is precisely defined, and because the bone marrow aplasia is irreversible and is not complicated by other side-effects of irradiation. This model is ideal for studying immediate autologous reconstitution, since chemotherapeutically induced marrow aplasia has the disadvantage that some of the chemotherapeutic agent may circulate for a varying period of time, and so modify the response of engrafted marrow.

The cause of death in the animals which were irradiated but not reconstituted was infection, with a survival period of 4-12 days. This correlates with an absolute loss of granulocytes and monocytes and reflects severe bone marrow damage. The finding that when one or both femora were shielded all the animals survived, established that the amount of marrow contained in one femur (11.0 × 10<sup>6</sup> nucleated cells) is adequate for reconstitution. A slightly more rapid rise occurred with a larger graft, obtained by using both femora (Fig. 2). However, other studies<sup>2</sup> have established that a graft of 1.7 — 4.0 × 10<sup>6</sup> nucleated cells per kilogram body weight is adequate, and that larger grafts appear to confer no significant further benefit. If the weight of our animals is taken into account, these observations accord well with published data.

The technique in which the femur was de-roofed was abandoned, since neither internal fixation nor acrylic cementing was suitable for stabilising the weight-bearing bone. The mortality from infection in some animals in this group was thought to be influenced by femoral fractures with local haematoma formation.

In the group where the entire marrow core was replaced intact immediately after irradiation, the low survival rate is clearly explained by the extensive necrosis of the graft which occurred, and which was demonstrated at post- mortem. The necrosis probably resulted from an inability to vascularise the graft sufficiently rapidly to sustain cell survival. In the 2 animals which survived, superficial colonisation with haematopoietic tissue occurred, but the unpredictability of this occurring contra-indicates the use of large blocks.

Of significance are the results in the animals in which intramedullary grafting was carried out using small particles. In this group, the survival rate approached that of the animals that were autologously transplanted by the intravenous route. Histological examination of the bone marrow in these animals proved that it was entirely normal. Death in this group was related to infection at a period before engraftment had taken place. As in the previous group, necrosis was observed, and although it was patchy in distribution, it is thought to have limited the delivery of nutrients essential for rapid engraftment. This finding raises a question about particle implantation as opposed to mononuclear reconstitution where, we assume, nutrition would not be in balance.

Of particular interest is the finding that the grafting of 2.4 × 10<sup>6</sup> nucleated cells from one femur led to more rapid recovery of peripheral blood values than when either one femur, or both, was shielded. With the shielding of the femur, normal architecture is retained, and a rapid return of bone marrow function could be expected. This paradoxical delay in recovery may be explained by the systemic effect of irradiation, the so-called abscopal effect.

These studies have led us to conclude that in the experimental animal intramedullary block grafting is feasible, but offers no advantage over the intravenous route. When these findings are extrapolated to man, the benefits of the intravenous route are further emphasised, since the problems of a surgical procedure in the thrombocytopenic and agranulocytic patient will pose a formidable challenge.

We acknowledge support from the South African Medical Research Council and the Harry Crossley Fund at the University of Cape Town.

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Improved Survival Mediated by *Corynebacterium parvum* Following Allogeneic Bone Marrow Transplantation in Rabbits

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The effect of *Corynebacterium parvum* on survival was studied in aplastic rabbits transplanted with allogeneic bone marrow cells. Twenty four hours after receiving 1200 rad of total body irradiation, NZW males were reconstituted from unrelated R females using bone marrow combined with spleen cells or with marrow alone. The effects of treating donor, or both donor and recipient, with *C. parvum* were contrasted.

Rabbits in Group I were infused with a mixture of bone marrow and spleen cells. In control animals (Group Ia) none of the 14 animals survived to 40 days. Similarly, there were no survivors among 17 animals in group Ib where only the donors were pretreated with *C. parvum*. When both donor and recipient received this agent (Group Ic) three of the 13 animals (23%) survived beyond 40 days.

Animals in Group II were infused with marrow cells alone. Of the controls (Group IIa), 3 out of 24 animals (12.5%) survived to 40 days; pretreatment of the donor with *C. parvum* (Group IIb) resulted in survival of 8 of the 18 animals (44%); when both donor and recipient received this agent (Group IIc), 16 of 26 animals (62%) survived beyond 40 days.

Both the incidence and the severity of histologically demonstrable graft-versus-host disease was markedly reduced in the surviving animals. These observations demonstrate a significant effect of parenteral *C. parvum* on the prolongation of survival in the irradiated rabbit following transplantation with allogeneic bone marrow cells, which is related to reduction in GVHD.

**Key words:** allotransplantation — rabbits — *Corynebacterium parvum* — prolonged survival — decreased GVHD

Two immunologic phenomena have been predominantly responsible for preventing the considerable potential of clinical bone marrow transplantation from being realized: first, graft rejection; and, second, the high morbidity and mortality occurring in graft-versus-host disease, which may take either an acute or chronic form in successfully transplanted recipients.
In man, both prophylaxis and treatment of established graft-versus-host disease are unsatisfactory. Clinical and histopathologic manifestations of this unique immunologic phenomenon are well recognized, and the many therapeutic regimens being evaluated reflect the controversy surrounding pathogenesis. Thus, the appearance of this syndrome in approximately 70% of patients matched with their donors at the major histocompatibility complex suggests that other loci may be involved in the immune recognition. Furthermore, uncertainty persists about the way in which tissue destruction is mediated (1), although it appears that aberrations in the T-cell population result in the development of autotoxic or toxic cells (2). The role of the monocyte-macrophage system in this reaction is also not clear, but there is at least experimental evidence suggesting that cytotoxic macrophages may have a role to play (3).

Not surprisingly, this uncertainty has resulted in studies designed to prevent or to treat graft-versus-host disease with methotrexate (4), cyclophosphamide (5), or other agents, including antilymphocyte serum (6). However, most of these have met with limited success. In experimental animals attempts to modify graft-versus-host disease have been successful. In a mouse spleen model the phenomenon has been abrogated in the F1 hybrid by pretreating the host with Corynebacterium parvum (C. parvum) (7). These studies were undertaken to define the effects of this agent in another species and to examine the prolongation of survival in aplastic rabbits reconstituted with allogeneic bone marrow and spleen cells or bone marrow alone.

MATERIALS AND METHODS

Rabbits. New Zealand White (NZW) and R strain animals, having an average weight of 2.0 kg were used throughout. The animals were individually housed and received water containing prophylactic sulphaquinoxaline sodium (Embazin, May and Baker, Dagenham, England), and a standard rabbit diet (Epol, Johannesburg) medicated with Amprolium (Merck & Co., Inc., USA). Temperature, weight, and full blood count with differentials were done twice a week.

Strain difference. In all experiments donors were R females and the recipients NZW males. Lack of suitable antisera prevented histocompatibility typing. Standard mixed lymphocyte cultures (8) to demonstrate strain differences were not uniformly successful. Using a modified technique (9), consistent antigenic disparity was demonstrated between the two strains, and was most striking when mitomycin-treated lymphocytes derived from mesenteric lymph node were used as antigen and appendicular lymphocytes as responding target cells. Results at day 3 give stimulation indices between 1:3.6 and 1:13.9.

In each experimental study identical donor and recipient combinations were used, and the results obtained with the modified mixed lymphocyte reaction were confirmed by means of skin graft acceptance or rejection. In 12 studies uniform rejection of allografts was present between 6 and 12 days (mean day 10); autografts were uniformly accepted without sloughing.

Irradiation. The rabbits were rendered aplastic by delivery of 1200 rad total body irradiation from a cobalt source 100 centimeters above the midplane of the prone animals at a rate of 50 rad/minute. We have previously demonstrated (10) that irradiation delivered in this way produces uniform aplasia without unacceptable radiation complications. Specifically, no gastro-intestinal tract lesions were demonstrated. We did not find any difference between this technique and that in which radiotherapy was fractionated, as has been suggested by other investigators (11,12).

Radiation controls. 28 animals received irradiation and were monitored without transplantation.

Reconstitution with bone marrow and spleen cells—Group I. These animals received a combination of bone marrow and spleen cells by intravenous infusion 24 h after irradiation. Group Ia (n = 14) were transplanted without additional
manipulation. Group Ib (n = 17) received cells from donors pretreated with 2.8–3.5 mg C. parvum intravenously on days –4 and –1 prior to sacrifice and cell collection. Group Ic (n = 13) received the cell mixture from donors pretreated as above, and the recipient received, in addition, the same dose of C. parvum intravenously on the third and sixth day post-transplantation.

Reconstitution with marrow cells alone—Group II. These animals received only bone marrow cells by intravenous infusion 24 h after irradiation. Group IIa (n = 21) were transplanted without additional manipulation. Group IIb (n = 18) received cells from donors pretreated with 2.8–3.5 mg of C. parvum intravenously on days –4 and –1 prior to sacrifice and cell collection. Group IIc (n = 26) received the cell mixture from donors pretreated as above and the recipient received, in addition, the same dose of C. parvum intravenously on the third and sixth day post-transplantation.

Corynebacterium parvum. Wellcome Coparvax, a formalin-fixed suspension of organisms, was administered intravenously in a dose of 2.8–3.5 mg/2 kg rabbit.

Procedure for cell collection from the donor. Marrow was obtained from the long bones of the donor animals who were sacrificed with a short acting barbiturate (Sagatal). The cells were rendered monocellular by passing them through a series of stainless steel screens as described (10). Rabbits received an intravenous infusion of 2.0–4.0 × 10^8 cells/kg. Where marrow and spleen combinations were used for transplantation, the same range of marrow cells were given, but an additional 0.71–1.3 splenic cells/kg were added before infusion; the spleen cells had also been passed through the screens.

Some variation in the number of donor cells infused was unavoidable. As the basis for a previous study (10) we have established that infusion of 1.8 × 10^8 cells/kg or above is on a plateau for survival and therefore not a variable in the study. In addition, curves for the return of peripheral granulocyte and platelet count were constant within that range of nucleated cells infused. Furthermore, at any given time interval histology and marrow hematopoietic tissue were similar, provided more than 2.0 × 10^6 cells/kg were grafted.

Trypan blue exclusion confirmed a 98% viability of the cells administered and differential count of the marrow showed a full range of hematopoietic tissues to be present in the graft.

Histology. At sacrifice, or when the animal died, detailed autopsy studies were carried out on tissue fixed in buffered 10% formal saline. Sections were prepared from representative areas and examined after staining with hematoxylin and eosin.

Statistics. Taking into account the relatively small numbers of observations and differences in numbers between groups, comparison of means was undertaken as for independent samples (13). The difference between the means of the two samples was calculated and probability assigned on the basis of the Student’s t test (14). Differences between the means were compared after 40 days survival since, from this point, survival curves were constant on a plateau.

RESULTS

Control animals. 28 animals, irradiated but not transplanted, died with a mean survival of 6.8 days (SEM ± 0.08) (range 2–11 days). The fall in peripheral granulocyte and platelet count reached a nadir between four and six days after the irradiation. Autopsy studies confirmed marrow aplasia, and cause of death was established as infection in the majority of animals. Minimal associated hemorrhage was present in the gastrointestinal tract of occasional rabbits. The characteristic histologic finding was bone marrow aplasia, widespread presence of colonies of bacteria in all the organs, and loss of lymphoid tissue through the gut, thymus and the spleen.

Radiation complications were excluded by autotransplantation in a series of rabbits, all of whom survived and regenerated normal peripheral counts and bone marrow as reported (10). Serial studies failed to show complications such as late infection, myelofibrosis or delayed bone marrow failure.

Transplanted animals

a) Survival curves. Animals which died within 7 days of allogeneic transplan-
tation are excluded from further consideration.

i. No C. parvum administration:
   At 40 days there were no survivors among 14 animals infused with a mixture of bone marrow and spleen cells (Group Ia), and 3 of 24 animals (12.5%) who received marrow alone (Group IIa) reached this end point.

ii. Donors pretreated with C. parvum:
   There were no survivors among 17 animals transplanted with a mixture of marrow and spleen cells (Group Ib); 8 out of 18 animals (44%) survived 40 days following reconstitution with marrow cells alone (Group IIb).

iii. C. parvum administration to both donors and recipients:
   Three of the 13 animals (23%) who received a mixture of bone marrow and spleen cells (Group Ic) survived 40 days, whereas 16 of 26 animals (62%) who received marrow alone (Group IIc) also survived 40 days.

The influence of C. parvum on animals being reconstituted with bone marrow and spleen cells is summarized in Fig I; there is no statistically significant difference in survival between these three sub-groups. A similar comparison is made in Fig 2 for the animals receiving marrow alone. There is no significant difference between control animals and those receiving cells from C. parvum-treated donors \( (0.1 < P < 0.5) \). In contrast, significance is achieved between the survival of control animals and those where C. parvum is administered to both donor and recipient \( (0.2 < P < 0.05) \). The difference between Group IIb and IIc is not significant \( (0.1 < P < 0.5) \).

Figure 1. Comparison of survival in groups of rabbits allotransplanted with a mixture of bone marrow and spleen cells. There is no significant difference between the three groups.

b) Hematology. The pattern for regeneration of peripheral granulocyte and platelet count was analyzed. No difference was evident between Group I and Group II and, furthermore, the subgroups in the two transplanted series were also comparable. There was no difference in hematological recovery between allografted or autografted animals receiving between 2.0 and \( 4.0 \times 10^8 \) nucleated cells/kg. Furthermore, these patterns closely approximated the regeneration of peripheral blood granulocyte and platelet count in animals where one or both femora were shielded during the radiotherapy.

c) Clinical syndrome. The allografted animals differed dramatically from autograft controls or where femoral shielding had been used. Gross loss of weight was the striking feature; at day 40 mean weights were 0.9 and 2.8 kg respectively. There was, in addition, patchy but typically extensive hair loss. Diarrhea was a variable feature, with cultures generally being negative. This profound wasting syndrome with the copious watery diarrhea was limited to those that received allogeneic marrow, and was not present in irradiated animals or in those undergoing autografting.
The cause of death was typically infection in the respiratory tract with nasal discharge of pus. Pneumonia was often present, with extensive destructive abscess formation being a feature. Macroscopic findings were generally unremarkable apart from the subcutaneous atrophy, reduction in spleen size, and rather friable bowel most obvious in the ileum.

d) Histopathologic studies. In all the animals a rising peripheral blood count was associated with histologically normal hematopoietic repopulation of the marrow.

Histology of the skin, gastrointestinal tract, and the liver were characteristic of graft-versus-host disease: in the skin, aggressor lymphocytes were present in the epidermis and spongiosis was present, particularly at the junction with the derma; in the gastrointestinal tract, loss of glands and patchy to total mucosal denudation were most striking in the small bowel; in the liver, infiltration of lymphocytes, loss of the limiting plates, and destruction of bile ducts by lymphocytes were present; in the spleen, lymph nodes and thymus, generalized lymphoid atrophy was evident. These features were similar to those reported in man (15) and other experimental animals.

DISCUSSION

The total body irradiation technique used in this animal model produces uniform aplasia of the entire bone marrow without complications such as the lethal gastrointestinal tract symptoms described by others (11,12,16). It is not known why 1200 rad as a single fraction should, in our studies, be associated with apparently less morbidity than fractionated doses used by others. Furthermore, the control rabbits in our study which were allografted with bone marrow alone and had no exposure to C. parvum, had 40-day survival of only 12.5%, in contrast to higher figures reported by others (17,18). This variation in results may be explained by two fundamental differences between the present study and others that we have cited. First, the radiation schedules differed: other investigators used fractions of 600 rad and, 24 h later, a further 500 rad, whereas, we used a single exposure of 1200 rad. It remains theoretically possible that the fractionated doses may have less completely ablated hematopoietic stem cells, thereby permitting a higher percentage of spontaneous hematopoietic reconstitution with survival. Secondly, in their studies a single strain of New Zealand White rabbit was used, although these were not apparently inbred. In contrast, we transplanted across a strain difference, and greater antigenic disparity may have contributed directly to lower survival in our untreated group consequent upon graft-versus-host disease.

The traditionally accepted cellular infiltrate found in graft-versus-host dis-
ease was not initially present in the organs of our successfully engrafted animals. As time elapsed, however, lymphocyte repopulation occurred and at 40 days the clinically evident wasting syndrome could be correlated with histopathologic changes of graft-versus-host disease. The spleens were initially atrophic but gradually regained their lymphoid follicles. Essentially similar findings have been described (17). Spleen size, although smaller than unirradiated animals, was comparable to that found following autologous bone marrow reconstitution. We have been unsuccessful in demonstrating the hemolytic anemia that has been a prominent feature in some of the other studies (18).

The rationale for infusing spleen cells together with bone marrow in some of the animals was to try to produce a more acute form of graft-versus-host disease; similar approaches have been described by others (19). Such a sub-set of animals would permit further characterization of any effects resulting from the administration of C. parvum. Notably, these animals did not differ in the rate of engraftment, the course of the post-transplantation wasting syndrome, or the histology of the organs when compared to animals reconstituted with bone marrow cells alone. Nevertheless, supplementary spleen cells do appear to influence the death rate from graft-versus-host disease. Thus, in these animals, C. parvum treatment of the donors is without effect and only minimal benefit accrues when both donor and recipient receive this agent (Group Ic), so that 23% of the animals survived for 40 days. In contrast, clear improvement in the survival rate occurs when C. parvum is given to animals allotransplanted with marrow cells alone. Here, survival is prolonged when only the donors receive this agent; this effect is much greater when, in addition, recipients receive the immunologic adjuvant on the third and the sixth day following marrow infusion.

It is noteworthy that in the latter series (group IIC), 9 of the 26 animals who reached 40 days have survived beyond 100 days. The explanation for further deaths in this group due to graft-versus-host disease may be related to the way in which the C. parvum is scheduled. Thus, continued administration of C. parvum beyond the first week following transplantation may be an effective means of prolonging survival.

The decision to examine the effect of C. parvum administration on graft-versus-host disease is an extension of the observation by Biocchi et al (7) that this agent effectively abrogated the syndrome in the parental F1 hybrid mouse combination. That model is not strictly comparable to the situation which occurs when allogeneic marrow is transplanted from one HLA identical and MLC non-reactive sibling to another; the studies in our rabbits partly resolve the issue. Thus, they differ in that we were not able to match donor and recipient on the basis of histocompatibility testing, for want of appropriate antisera. Parallel with that clinical situation, however, there is, except in the case of identical twin transplants, genetic disparity between donor and recipient as the basis for graft-versus-host disease, although not at the major histocompatibility complex.

A further reason for selecting C. parvum for study are observations that T-lymphocytes are involved in the process of graft-versus-host disease and that this immunologic agent affects this
population (20) via interaction with the macrophage (21,22). Furthermore, *C. parvum* is known to be a potent stimulator of the reticuloendothelial system and to have adjuvant and antitumor activity (23–26). The central role of the macrophage, and the way it is influenced by *C. parvum*, is supported by observations (27) that the antitumor effect of this agent can be annulled by using antimacrophage sera. Furthermore, Bash (28) demonstrated that a T-lymphocyte sub-population is required in the inductive phase of macrophage activation by *C. parvum* and that subsequent suppression of this specific cell function is mediated by these macrophages.

*C. parvum* has been shown in our experimental model to have a clear effect in prolonging survival of irradiated rabbits reconstituted with allogeneic bone marrow cells, even when only the donor is treated prior to transplantation. Although only two doses are used, it is notable that this agent has a much more profound benefit when donor treatment is combined with its administration to the recipient after transplantation. In contrast, *C. parvum* has a lesser effect in prolonging survival of rabbits when spleen cells are transfused together with the marrow. At the present time it is not clear how *C. parvum* brings about improved survival in this model system. Theoretical possibilities include a direct influence on the macrophage or, more probably, modification of T-lymphocyte function in pathogenesis of graft-versus-host disease.

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C. PARVUM ABROGATES GVHD


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Brief Communication

THE LYMPHOCYTE: MONOCYTE RATIO: B- AND T-CELL RATIO AFTER RADIOThERAPY, CHEMOTHERAPY AND SURGERY†

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Differential white blood cell counts were taken from 42 previously untreated patients with mammary carcinoma who received radiotherapy. 10 patients with Stage III mammary carcinoma who received chemotherapy, 19 patients previously treated by a simple mastectomy, and 4 untreated patients. The percentage of B- and T-cells in the peripheral blood also was measured. Severe lymphopenia was seen in patients treated with radiotherapy and chemotherapy. The percentage of B- and T-cells remained constant before and after radiotherapy, and there was no selective depression of T-cells. The monocyte count, 3 months after radiotherapy, was significantly higher than the pre-treatment mean count. The percentage of B- and T-cells in the peripheral blood remained unchanged before and after treatment with chemotherapy. The mean lymphocyte counts following surgery were slightly higher than the control values whereas the monocyte counts were slightly lower than the control values. Surgery did not appear to influence the percentages of B- and T-cells in the peripheral blood.

EAC and E rosettes (B- and T-cells), Monocyte lymphocyte ratio, Radiotherapy, Cyclophosphamide, Surgery, Fractionation, Mammary carcinoma.

INTRODUCTION

Stjernwärd et al.1 reported a selective depletion of T-cells in the peripheral blood following radiation therapy for mammary carcinoma, as well as a long lasting lymphopenia. Turk and Poulter2 reported a selective depletion of B-cells in the lymph nodes of mice following large single doses of cyclophosphamide. This finding suggested a way to depress B-cells selectively in humans with mammary carcinoma. If B-cells could be selectively depressed, immunoglobulin formation and “enhancing” antibodies could possibly be suppressed.3 We were also interested in the killing ability of B-cells following cyclophosphamide. Dowdle et al.5 reported impaired B-cell killing ability in leukemic patients, and we were interested to see if this was true in patients with mammary carcinoma. Rotman et al.6 reported that monocytosis occurred in patients on radiotherapy. They used a sophisticated electronic counter that could count 10,000 cells at a time. Although our differential counts were still done by hand, our data tends to corroborate the findings of Rotman. In addition, an inverse relationship between the monocyte and the lymphocyte count could be illustrated following chemotherapy, radiotherapy or surgery.

Finally, we could not substantiate previous reports4 that the lymphocyte count decreased with increasing tumor burden. Our patients with advanced mammary carcinoma were not lymphopenic, with the exception of 5 patients with so-called inflammatory carcinoma (cancer en cuirass).

METHODS AND MATERIALS

Radiotherapy: Mammary carcinoma

We took differential white cell counts before radiotherapy from 42 untreated patients with various stages of mammary carcinoma; we took them again immediately after completion of radiotherapy and at 3 monthly intervals thereafter.

White cell counts were done by electronic counter

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in the routine hematology laboratory and the
differential counts were done manually by counting
100 cells.

The total lymphocyte and monocyte counts were
calculated by multiplying the percentage of these
cells by the total number of white cells per cubic ml
of blood; this total was divided by 100.

EAC and E rosette forming cells (B- and T-cells)
20 ml of heparinized blood was taken by venipunc-
ture. Preservative free heparin was used. Lym-
phocyte separation was done by the method of
Böyum, Thorsby and Brattie. EAC and E rosette
forming cells were determined by the methods des-
cribed by Jondal et al. and Bianco et al.

Radiotherapy: cervical carcinoma
In order to determine the effects of radiotherapy on
the lymphocyte and monocyte counts during the first
4 weeks of treatment, we did differential white blood
cell counts on 29 patients with carcinoma of the
cervix prior to treatment and weekly thereafter for 4
weeks. Since 3 fractionation schemes were used, we
analysed the data in terms of fractionation as well.
The 3 schemes used were: twice weekly fractions of
600 rad each to a total dose of 3600 rad; twice weekly
fractions of 400 rad each to a total dose of 4000 rad;
and 2 fractions of 200 rad daily to a total dose of
5500 rad. The field arrangement for the 600 rad frac-
tions was by 2 parallel opposing fields, 15 cm²; for the
other 2 fractionation schemes, the so-called 4 field
"box" technique was used. ⁶⁰Co at a source to skin
distance of 80 cms was used in each case.

Chemotherapy
Ten previously untreated patients with stage III
mammary carcinoma had differential white blood cell
counts taken prior to, and for 3 consecutive weeks
after treatment with a single intravenous dose of
cyclophosphamide (50 mg per kg). Peripheral B- and
T-cells were determined before and 3 weeks after
treatment. B-cells were determined by their ability to
form rosettes (EAC rosette forming cells) as well as
an immunofluorescent method.

The B-cell killing ability of the lymphocytes were
determined before and after treatment by the method
described by MacLennan and was expressed as the
effect on target cell ratio to achieve 50% cell kill
after 19 hr incubation.

Surgery
19 Patients who had simple mastectomies pre-
viously had differential white cell counts taken at
intervals from 1 week to 6 months after surgery. EAC
and E rosette forming cells were determined as well.
The mean lymphocyte and monocyte count of 42
untreated patients with mammary carcinoma was
used as controls.

Radiation technique for mammary carcinoma
The 42 patients previously untreated, had mam-
mary carcinomas of varying stages and were there-
fore treated by 1 of 3 methods:
⁶⁰Co: A single irregular anterior field was used to
cover the internal mammary and supravacular
lymph nodes. Daily fractions of 200 rad for 5 days
each week to a total given dose of 5500 rad at 80 cm
source to skin distance (SSD) was used. This tech-
nique was used for post mastectomy patients with
negative apical lymph nodes.

Orthovoltage: 250 K V, 3.5 mm Cu half value layer
HVL at 50 cm SSD. For patients with metastatic
disease, 2 palliative tangential fields to the affected
breast were used. We attempted to deliver a skin dose
or central dose of 4500 rad. The field sizes were
usually 20 × 10 cm. The fractionation scheme used
was 200 rad daily for 5 days. For patients with apical
lymph node involvement, or other signs of Stage III
disease, a 5-field technique was used. This included a
sternal field, usually 20 × 7.5 cm, 2 tangential fields
with or without supplementary fields and 2 parallel
opposing fields to the supravacular area. We
attempted to deliver a central dose of 4500 rad; if this
was not possible, the given dose would be taken to
skin tolerance, usually 4500 rad.

The lymphocyte count as a function of the tumor
burden (stage) in patients with mammary carcinoma
The lymphocyte counts of 38 healthy females were
compared to 22 Stage I, 20 Stage II, 36 Stage III and
30 Stage IV patients with mammary carcinoma, and 5
patients with inflammatory carcinoma (Table 1).

RESULTS
Radiotherapy: Mammary carcinoma
The lymphocyte count and monocyte counts in 42
patients following a full course of radiotherapy ap-
ppears in Fig. 1...
Table 1. The influence of breast cancer stage on the lymphocyte count

<table>
<thead>
<tr>
<th>(Normal controls) 38 healthy patients</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
<th>Inflammatory carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Means</td>
<td>2021 ± 580</td>
<td>2127 ± 170</td>
<td>2149 ± 691</td>
<td>2287 ± 892</td>
<td>2273 ± 681</td>
</tr>
<tr>
<td>Number of patients</td>
<td>38</td>
<td>22</td>
<td>20</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>t-test</td>
<td>—</td>
<td>0.7</td>
<td>0.12</td>
<td>1.60</td>
<td>1.67</td>
</tr>
<tr>
<td>Significance</td>
<td>N.S.¹</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>p &lt; 0.005</td>
</tr>
</tbody>
</table>

¹N.S. = not significant.

Table 2. Monocyte and lymphocyte counts as a function of time after radiotherapy or surgery in patients with mammary carcinoma

1. Monocyte count

<table>
<thead>
<tr>
<th>Time (weeks) after treatment</th>
<th>1</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
<th>Total no. of counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>330 ± 167</td>
<td>339 ± 234</td>
<td>287 ± 90</td>
<td>317 ± 163</td>
<td>338 ± 175</td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>445 ± 140</td>
<td>391 ± 231</td>
<td>656 ± 150</td>
<td>476 ± 254</td>
<td>360 ± 156</td>
<td></td>
</tr>
</tbody>
</table>

2. Lymphocyte count

<table>
<thead>
<tr>
<th>Time (weeks) after treatment</th>
<th>1</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
<th>Total no. of counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>1688 ± 587</td>
<td>2664 ± 806</td>
<td>2311 ± 1703</td>
<td>2264 ± 298</td>
<td>2108 ± 877</td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>381 ± 119</td>
<td>1107 ± 292</td>
<td>1477 ± 538</td>
<td>1574 ± 502</td>
<td>1682 ± 776</td>
<td></td>
</tr>
</tbody>
</table>

The mean lymphocyte count for 42 previously untreated breast patients was 2223 ± 703; monocyte count 332 ± 163. The numbers in the right hand columns are the number of counts done at each interval.

Radiotherapy: Cervical carcinoma

The lymphocyte count following radiotherapy for cervical carcinoma follows the same pattern as that seen in the breast cancer patients. In this small longitudinally followed series, the monocyte count was also depressed in the first 4 weeks of treatment with our conventional counting method although the lymphocyte count was again significantly depressed at all points and fell from pretreatment mean values of 2318 ± 565 to 584 ± 307 2 weeks after the start of radiotherapy (p < 0.001; Fig. 2).

The influence of 3 different fractionation schemes is interesting and suggests that small daily fractions are more damaging to the lymphocyte population than large fractions given twice weekly. This remains true if the lymphocyte counts for the various fractionation schemes are compared at equivalent TDF (time dose fractionation) values. For TDF values of 33, e.g. the lymphocyte count for 5 fractions of 200 rad each has a mean value of 547 ± 289 compared to 1065 ± 481 and 1904 ± 350 for the 2 large fractionation schemes respectively (0.01p < 0.001; Table 4).

Fig. 1. The influence of radiotherapy on the lymphocyte- and monocyte count in patients with mammary carcinoma as a function of time—the first 6 months. Forty-two previously untreated patients were followed longitudinally.¹

¹The same patients originally seen were followed in time.
Table 3. EAC and E rosette forming cells (B- and T-cells) after radiotherapy in patients with mammary carcinoma

<table>
<thead>
<tr>
<th></th>
<th>B-cells (EAC) %</th>
<th>Total</th>
<th>T-cells (E) %</th>
<th>Total</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before radiotherapy</td>
<td>33.4</td>
<td>742</td>
<td>64.0</td>
<td>1422</td>
<td>42</td>
</tr>
<tr>
<td>After radiotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediately</td>
<td>31.7</td>
<td>176</td>
<td>65.5</td>
<td>364</td>
<td>30</td>
</tr>
<tr>
<td>3 months</td>
<td>34.1</td>
<td>422</td>
<td>67.0</td>
<td>829</td>
<td>20</td>
</tr>
<tr>
<td>6 months</td>
<td>27.8</td>
<td>370</td>
<td>57.4</td>
<td>764</td>
<td>10</td>
</tr>
</tbody>
</table>

Fig. 2. The influence of radiotherapy on the lymphocyte and monocyte count in patients with carcinoma of the uterine cervix as a function of time—the first 4 weeks. Twenty-nine previously untreated patients were followed longitudinally.

Chemotherapy: Total white cell count

After 50 mg/kg cyclophosphamide intravenously, the total white cell count dropped significantly ($p < 0.005$) from pre-treatment values of $7759 \pm 1864$ to $3271 \pm 1690$ at the end of the first week and $4200 \pm 2474$ at the end of the second week. The count was $8380 \pm 390$ at the end of the third week, higher than the starting value (overshoot phenomenon).

The lymphocyte count

The total peripheral lymphocyte count dropped significantly ($p < 0.005$; Fig. 3), from a pre-treatment mean value of $2090 \pm 114$ to $875 \pm 622$ at the end of the first week. At the end of the third week the mean lymphocyte count was $1072 \pm 684$. This value was still significantly depressed compared to the starting value ($p < 0.025$; t-test).

The monocyte count

From pre-treatment values of $527 \pm 187$ the mean value for the monocyte count dropped significantly ($p < 0.005$; Fig. 3) to $279 \pm 304$ at the end of the first week and overshot significantly to values of $833 \pm 400$ ($p < 0.01$) at the end of the third week.

B- and T-cells (EAC and E rosette forming cells)

The B-cells formed 34% of the peripheral lymphocytes before cyclophosphamide and 31% 3 weeks after. This percentage difference was not significant. Turk and Poulter reported selective depletion of the B-cell areas in lymph nodes of mice following large single doses of cyclophosphamide.

In the human patient with mammary carcinoma, however, there is no selective depletion of the B-cells in the peripheral blood. We did not study lymph node morphology. The percentage of B-cells registered in the peripheral blood prior to treatment, by the immunofluorescent method was 34, 14%; at the end of 3 weeks this method identified 50.16% of the peripheral blood lymphocytes as B-cells. This difference was statistically significant ($0.01 < p < 0.05$; t-test). T-cells formed 64% of the peripheral lymphocyte count prior to treatment with cyclophosphamide; 3 weeks after, they accounted for 55%. This percentage drop was not statistically significant.

B-cell killing ability

The effect to target cell ratio for 50% cytotoxicity at 19 hr incubation for control (normal healthy
females) lymphocytes ranged from 1.2 to 4.2 with a mean value of 3.78. Before cyclophosphamide the range in 10 patients with Stage III mammary carcinoma was 1.3 to 6.4 with a mean value of 3.7. Three weeks after 50 mg/kg of cyclophosphamide the mean value was 3.70. These values do not differ significantly from those of healthy controls.

Impaired lymphocyte killing ability in this laboratory was seen in 7 patients with chronic lymphocytic leukemia (E: T50 value of 36.3), as reported by Dowdle et al.2

Surgery

Differential white cell counts were taken from 19 patients who previously had mastectomies. The mean lymphocyte and monocyte counts from 42 previously untreated patients with mammary carcinoma were taken as baseline values.

Following surgery the lymphocyte count did not appear to be affected (Fig. 4), except for the low point at 2 weeks post-operatively. Thereafter it remained at normal or slightly higher than normal values for 6 months or longer (Table 2).

The monocyte count dropped to values slightly lower than normal after surgery (Fig. 4, Table 2). B- and T-cells expressed as a percentage of the peripheral lymphocytes had a mean value of 32±13 and 62±12 respectively, compared to a value of 33.4±10.8 and 64±10.3 respectively in 42 previously untreated patients with mammary carcinoma.

The lymphocyte count and tumor burden (stage)

We could not find any correlation of the tumor burden with the lymphocyte count in 108 patients with various stages of mammary carcinoma. The exception was the mean lymphocyte count in 5 patients with inflammatory carcinoma. The mean count in these 5 patients was significantly depressed (Table 1).

DISCUSSION

Meyer10 states that radiotherapy induces a lymphopenia which may last for years; our data collected on patients who had radiotherapy for mammary carcinoma many years previously agrees with this finding (Table 2).

The induced lymphopenia is maximal at the end of a course of radiotherapy for mammary carcinoma and drops to 381/2223 or 18% of the pre-treatment values; 96 weeks later the lymphocyte count is still only 1682/2223 or 77% of the original mean values.

In radiotherapy for cervical carcinoma, the degree of lymphopenia after the completion of a course of radiotherapy depends on the fractionation scheme used (Table 4). At equal T.D.F. values, the lymphocyte count dropped to 23% of starting values for small fractions, 64% and 65% for the large fractionation schemes respectively. A possible explanation for this phenomenon is repopulation of the lymphocytes between fractions.

Chemotherapy (cyclophosphamide) likewise induces a lymphopenia which is maximal (42% of baseline values) 1 week after a single large dose (50 mg/kg). Three weeks later, there is still a significant lymphopenia (53%).

Surgery, on the other hand, does not seem to have any long term effects on the lymphocyte count (Fig. 4, Table 2); if anything the lymphocyte count is higher than baseline values.

Papetetous et al.15 reported that a larger percentage (57%) of Stage I patients had lymphocyte counts over 2000 when compared to Stage III patients (48%).

Our data does not confirm this finding. Of 108 patients tested, 53% of Stage I, 59% of Stage II, 51% of Stage III and 67% of Stage IV patients had lymphocyte counts over 2000. Papetetous has chosen 2000 as criterion because that was the approximate mean lymphocyte count for healthy control patients. Our own control (healthy) patients had a mean lymphocyte count of 2050. The mean lymphocyte counts in our patients with mammary carcinoma does not decrease with increasing tumor burden (stage of disease, Table 1). The exception is for 5 patients with inflammatory carcinoma (cancer en cuirass), where there is significant lymphopenia (p<0.005, t statistic).

Rotman et al.14 reported that therapeutic irradiation induces monocytosis in the patients so treated, and that they could tell whether a patient was receiving radiotherapy merely by observing a series of differential blood counts. They used a sophisticated new counting method. Our data for patients with mammary carcinoma who had radiotherapy, supports

Fig. 4. The influence of surgery on the lymphocyte and monocyte count as a function of time—the first 6 months. Nineteen patients were followed horizontally.‡

Incidental patients investigated some time, at random, after an event.
this finding even though we used the old hand-counting method (Fig. 1, Table 2). However, we also observed a mononcytosis 3 weeks after a single large dose of cyclophosphamide (Fig. 3) so that this finding (monocytosis in the presence of a simultaneous lymphopenia) may not be peculiar to radiotherapy; it would be interesting to see the monocyte count followed by the more sophisticated counting method described by Rotman et al.41 in patients on chemotherapy.

Following surgery, the monocyte count tends to remain lower than baseline values (Fig. 4, Table 2). In general, looking at the previously mentioned Figs. (1, 3, 4) there seems to be an inverse relationship between the monocyte and lymphocyte counts; is there a homeostatic stimulus operative following radiotherapy that is fully successful for the monocyte and only partly for the lymphocyte? Does radiotherapy cause a reduction in the number of lymphocyte stem cells capable of responding, so that the remaining ones, maximally stimulated, cannot repopulate the lymphocytes to pre-treatment levels, even years afterwards? Does radiotherapy release factors into the bloodstream with a long half life that "sets" the lymphocyte "norm" at new low levels, and at the same time allows the monocyte count to stabilize at higher levels?

Despite these impressions, the monocyte count fell despite the lymphopenia induced following radiotherapy for cervical carcinoma (Fig. 2). This could result from the relative inaccuracy of the hand-counting method for monocytes, or perhaps it could represent a different reaction of the monocytes to cervical carcinoma, compared to mammary carcinoma. Here again, a repetition of this type of data with a better counting method for monocytes may be of value.

Turk and Poulter5 reported a selective depletion of the lymphocytes from the B-cell territories in the lymph nodes and spleen of the mouse after single doses of cyclophosphamide. Sobolovic et al. showed that in rabbits, cyclophosphamide reduced the lymphocyte number in all organs considerably, with maximal reduction in the appendix. The lymphocytes that disappeared were Ig positive cells with slow electrophoretic mobility (B-cells).

Dumont6 likewise reported B-cell depletion after cyclophosphamide treatment in the mouse.

These findings were of considerable interest, since coating with blocking antibodies in vivo on tumor cells may be one explanation of the non-stimulability of autochthonous peripheral lymphocytes by the patient's tumor cells, by suppressing B-cell activity, the production of "enhancing" antibodies may be stopped.

Following a single large dose of cyclophosphamide (50 mg/kg) given as a bolus, 10 patients with advanced (Stage III) mammary carcinoma were assessed.

The cyclophosphamide induced a leukopenia, lymphopenia, as discussed. We did not study lymph node morphology, but determined the patients' peripheral B-cells by the EAC rosette forming method, as well as the immunofluorescent method. We could not demonstrate any selective depletion of the peripheral B-cell population by either method; if anything, the percentage of the peripheral B-cells was increased when measured by the immunofluorescent technique.

Functionally, the ability of the B-cells to kill sensitized target cells did not change before and after cyclophosphamide therapy. We used the effector to target cell ratio for 50% cytotoxicity after 19 hr incubation as an index of B-cell killing ability.

Stjernswärd et al.8 reported a selective depletion of the T-cells following radiotherapy for mammary carcinoma.

Our data did not confirm this finding: a possible explanation may be that we extracted all phagocytic cells by means of a magnet and carbonyl iron; these cells have C3 receptors and could have been counted.
as B-cells. Stjernswärd et al. did not use this technique for extracting phagocytic cells.

What is the clinical relevance of the prolonged lymphopenia following radiotherapy? The controversy whether radiotherapy for operable mammary carcinoma leads to increased mortality is not settled yet; the fact remains that we have an altered set of circumstances after radiotherapy; the "milieu interieur" is disturbed, and the consequence of this disturbance remains to be fully assessed.

REFERENCES


DEFECTIVE B CELL KILLING BY LEUKAEMIC LYMPHOCYTES

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The expanded population of peripheral blood lymphocytes (PBL) in most patients with chronic lymphocytic leukaemia have, to a greater or lesser degree, the characteristics of bone marrow-derived lymphocytes (B cells). Since antibody-dependent cytotoxicity by uninduced mononuclear cells has been shown to be a function of a cell-mediated mechanism, this property has been attributed to B cells. We have studied the ability of blood lymphocytes from 7 patients with chronic lymphocytic leukaemia to participate in this so-called 'B cell killing'. Normal or leukemic lymphocytes (effector cells) were purified and freed of phagocytic cells with carbonized iron powder, magnetic separation and centrifugation on a ficoll-hypaque density gradient. Chicken erythrocytes (target cells) were labelled with 51Cr and sensitised by incubation, at optimal concentration, with rabbit anti-chicken erythrocyte Ig antibody. Effector cells were incubated with target cells at four different effector:target cell (E:T) ratios of approximately 16:1, 8:1, 4:1 and 2:1 and cytotoxicity was assessed by 51Cr release after 1 hour and 19 hours' incubation in RPMI-1640 tissue culture medium. The E:T ratio required for 50% cytotoxicity (E:T 50) was determined from the linear relationship between the log of the E:T ratio and the log of percent cytotoxicity, and was used as an index of cytotoxic effectiveness. E:T ratios for normal lymphocytes ranged from 12:1 to 88 (mean 30) after 1 hour and from 2.3 to 4.4 (mean 3.1) after 19 hours' incubation. Corresponding values for leukemic lymphocytes ranged from 88 to 4,600 (mean 930) after 1 hour and from 15.1 to 67.3 (mean 36.3) after 19 hours. The results indicate that chronic leukemic lymphocytes are defective in their ability to mediate antibody-dependent cytotoxicity.

T AND B LYMPHOCYTES IN BABOONS AND VERVET MONKEYS

P. BRAIN, Natal Institute of Immunology, Durban

Recent work has shown that in certain auto-immune diseases of man the T-B lymphocyte ratio is disturbed and the suppressibility of T lymphocytes with a standard antilymphocyte serum is altered. With the aim of ultimately investigating lymphocyte function in autoimmune primate disease, which auto-immune thyroiditis has been artificially induced, we studied the lymphocytes of normal baboons and vervet monkeys. T lymphocytes were detected by rosette formation, and B lymphocytes, by the formation of erythrocyte rosettes, as in man. Purification of these lymphocytic populations was achieved by placing the lymphocytes of normal baboons and vervet monkeys on a ficoll-hypaque density gradient. The purified lymphocytes were then incubated with autologous rabbit antibody and complement. The results indicate that baboon lymphocytes are unaltered, whereas vervet lymphocytes, especially B lymphocytes, are activated. The activation of B lymphocytes in vervet monkeys may be due to a priming effect of the induced autoimmune disease.

INTERRELATIONSHIP BETWEEN HL-A AND MIXED LYMPHOCYTE CULTURE (MLC) REACTIONS

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Provincial Blood Grouping Laboratory, Cape Town

We have studied the MLC reactivity in HL-A-identical unrelated individuals and in families with various degrees of consanguinity in a further attempt to demonstrate the genetic
Immunosuppressive therapy in bone marrow aplasia: The stroma functions normally to support hematopoiesis

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Abstract
In aplastic anemia (AA) patients responsive to antilymphocyte globulin (ALG) therapy, abnormalities in both stroma and progenitor cell (PC) pool have been described. The relevance of each pathophysiologic defect was characterized in 16 individuals, and data were compared to results from seven normal volunteers. Bone marrow mononuclear cells were split into two fractions. Stromal layers (SL) were prepared from the first, and a CD34+ enriched population was obtained by immunomagnetic selection from the second. In cross-culture experiments, 1×10⁶ of the latter from patients or controls were seeded on preformed SL, and adhesive PC were scored for the formation of blast colonies (CFU-BI) on day 5 of culture. Nonadherent progenitors were recovered and quantitated in a standard clonogenic assay (CFU-GM). There were significantly fewer CD34+ cells in the AA group (median 0.65%, SD 0.39%, vs. 1.62%, SD 1.4% p = 0.002). No morphological or cytologic differences between normal and aplastic SL were detected. Both equally supported the growth of CFU-BI from normal progenitors (mean 117, SD 20.4, and 103.1, SD 30.4), while this was reduced for the aplastic PC (mean 41.06, SD 42.9; p = 0.0002, exact two-tailed test). Similarly, the AA nonadherent PC had a decreased CFU-GM growth (mean 142.6, SD 104.8, vs. mean 361.7, SD 91.3), with a lower total clonogenic output (p = 0.0009). We conclude that aplastic stroma appropriately supports the growth of normal progenitors, whereas the depressed clonogenicity of the corresponding population derived from AA is unrelated to their attachment to SL but intrinsic to the CD34+ cells, whether adherent or not.

Key words: Aplastic anemia—Bone marrow stroma—Antilymphocyte globulin

Introduction
In adult mammals, hematopoiesis occurs in the bone marrow, where both myeloid and lymphoid lineages differentiate from common pluripotent stem cells and then mature into their respective functional elements. The steady-state production of blood cells reflects a dynamic interaction between positive and negative regulators secreted by the stroma [1–5], acting through specific receptors [6,7]. Additionally, a paracrine modulation of this system [8–10] has been described, constitutive or induced in response to indirect signals [1], such as release of M-CSF or G-CSF following the exposure of monocytes to interferon-γ, interleukin-1 (II-1), or endotoxin [2]. Further regulatory effects are exerted by cells of the lymphoreticular system, which are abundantly represented as an integral component of the microenvironment [11–13]. It is therefore not surprising that alterations in the immune response can affect hematopoiesis.

The pathophysiologic lesion in aplasia is unknown, but data from genetically anemic mice models (SS) and W/W mutations) [14] or from in vitro studies have localized the abnormality to the stem cell pool [15,16] or the myeloid microenvironment [17–19].

Clinically, aplasia may reverse after immunosuppressive therapy [20] or when autologous reconstitution follows allotransplantation. The mature cells are functionally abnormal [21,22], however, and a subgroup of patients evolve to paroxysmal nocturnal hemoglobinuria (PNH), myelodysplasia, or acute leukemia [23,24]. In vitro studies have shown that clonogenic potential is reduced, suggesting damage to the progenitor population [15] or their unbalanced interaction with the supportive stroma [25–27]. Similarly, in long-term bone marrow culture systems [28,29], formation of the adherent layer is reported to be suboptimal [30], while further studies have suggested that the primary defect resides in the stem cell [15,16].

In a modification of this system that exploits the adhesive properties of these progenitors, it is possible to examine an early population of undifferentiated blasts (CFU-BI) [31], which resemble murine spleen colony-forming cells (CFU-S) in their resistance to cycle-specific cytotoxins [32]. These precursors are capable of self-renewal, as demonstrated by the production of secondary blast colonies [31,33]. Applying this model, we have previously reported that the stroma derived from untreated AA patients or those responding to immunosuppression supports normal precursor growth effectively, but the hematopoietic progenitors are defective [34]. We have now extended these studies and confirm that the growth of stroma adherent and nonadherent CD34+ cells is reduced, adding support to the concept that the primary defect resides within the hematopoietic stem cells.

Patient population
Thirty-four individuals with severe aplasia [35] were referred for therapy; one had rapid improvement following a thera-
peutic abortion, and the remainder received ALG and high-dose methylprednisolone (HDMP) [36], followed by oxymetholone maintenance for a period of 6–9 months.

Twenty-three fulfilled our criteria for response, defined as freedom from transfusions, granulocyte count of >1×10⁹ and platelets of >40×10⁹; at a median of 1250 days, 65% were alive. From this group, a cohort of 16 patients and seven controls gave written informed consent to donate bone marrow for a study approved by the Ethics and Research Committee of the University of Cape Town and Groote Schuur Hospital.

Among these 16 individuals, aplasia was secondary to infectious hepatitis in one, following chloroquine intake for rheumatoid arthritis in two, associated with pregnancy in another, and idiopathic in the remainder. Nine were females; the median age was 33 years (range 17–61). At a median follow-up period of 4 years (range 1–7), the median performance status was 0 (World Health Organization). During that time, six patients relapsed and three required further immunosuppression—two of these are currently in partial response and receiving cyclosporin combined with androgenic corticosteroid maintenance. Two patients in complete response developed clinical features of PNH and a positive Ham's test. At the time of study and a median of 4 years (range 1–8) from therapy, the median hemoglobin was 134.5 g/L (SD 27.2), MCV 98 fl (SD 7.17), granulocytes 2.21×10⁹/L (SD 1.89), lymphocytes 1.53×10⁹/L (SD 0.71), monocytes 0.23×10⁹/L (SD 0.18), and platelets 127×10⁹/L (SD 80.44). Morphology of the bone marrow revealed variable degrees of dysplasia and dyserythropoiesis in 15 individuals. Cytogenetic analysis of the myeloid cells demonstrated chromosomal breaks and gaps in five of the 15 in 13–40% of metaphases. No specific translocations or other cytogenetic abnormalities were noted.

Methods

Bone marrow cells

The study group was represented by 16 patients who had responded to prior immunosuppressive therapy. Their bone marrow was aspirated under pethidine hydrochloride analgesia, 1 mg/kg, given 30 minutes before local anesthesia. In hematoologically normal controls, samples were obtained at sternotomy during cardiac surgery under general anesthesia.

Normal or aplastic mononuclear cells recovered from polysucrose diatrizoate density gradients (1.077 g/mL; Histopaque; Sigma, St. Louis, MO) were washed three times in a minimal essential medium (α-MEM) (Gibco, Life Technologies, UK) and resuspended in 10 mL of the same medium. Aliquots were taken for morphological assessment, indirect immunofluorescence, and quantitative determinations with an electronic particle counter (Multisizer, Coulter Electronics) and divided into two fractions, one for the establishment of adherent stroma and the other for the selection of the progenitor population.

Bone marrow stromal layers

Throughout the study, sequential cultures of stroma from three hematologically normal males ages 34, 41, and 42, who were undergoing surgery for coronary artery bypass grafting, were employed and compared to the aplastic SL in the morphological and functional studies. Neither the patients nor the controls had a history of recent inflammatory or infectious disorders.

Light-density bone marrow cells (LDBMC) were suspended in α-MEM containing 12% each of horse serum (HS) (Gibco, UK) and fetal calf serum (FCS) (Gibco), supplemented with hydrocortisone 2×10⁻⁶ mol and gentamycin 5 μg/mL. Cells were cultured in 33-mm petri dishes (T.C. dishes; Bibby-2500B, UK) at a concentration of 0.5×10⁶ per dish at 37°C in a humidified atmosphere containing 5% carbon dioxide, until the stromal cultures became confluent. The medium was changed once a week throughout the culture life. Stromal layers were inspected every 7 days, time to culture confluence was noted, and determinations of the area covered by fat cells were recorded.

Cytotoxic staining of the stroma

Upon confluence, representative cultures were air-dried and stained in situ with May-Grünwald-Giemsa [37] or incubated with 1 mL 0.1% bovine trypsin (Difco, Detroit, MI) for 10 minutes at 37°C, inactivated with cold FCS, and washed twice in α-MEM. Cytosplasts of the complete stromal suspensions were prepared (Cytospin 2; Shandon, UK) and stained with May-Grünwald-Giemsa, Sudan Black, Oil Red O, or alkaline or acid phosphatase. Differential counts of marrow stromal populations were performed on 300 cells, and results from patients and controls were compared.

Progenitor cell selection

The second fraction of LDBMC from normal individuals or aplastic patients was suspended in α-MEM containing 1% FCS, placed into 15×220-mm plastic dishes (Bibby; Gibco) and incubated for 2 hours at 37°C to remove adherent cells. The nonadherent population was lymphocyte-depleted with 100 μL CAMPATH 1M (6.25 mg/mL; 0.01 mL antibody/5×10⁶ cells) [38] in the presence of 30% fresh AB serum as a source of complement for 50 minutes at 37°C.

The resulting lymphocyte- and monocyte-poor progenitor cell concentrates were washed three times in medium, resuspended in 0.2 mL of cold (4°C) phosphate-buffered saline (PBS) containing 0.02 mL/10⁵ cells of antihuman CD34 antigen murine monoclonal antibody (Anti HPCA-1, My 10; Becton Dickinson, Sunnyvale, CA) [39], and incubated on ice for 30 minutes. Labeled cells were selected with paramagnetic beads covalently bound to affinity-purified goat IgG against all mouse IgG subclasses (M-450, product no. 125.01; Dynal, Oslo, Norway) [40]. Magnetic spheres were removed by suspending cells in 0.1 mL PBS containing 0.01 mL of a polyclonal murine immunoglobulin (Detachabead; Dynal, Oslo, Norway) [41], cell viability determined by trypan blue exclusion, cells counted in a Neubauer chamber, and numbers adjusted to a concentration of 1–5×10⁶/mL. Aliquots of cells obtained from the initial density gradient and from the final separation were incubated overnight at 37°C in medium containing 15% FCS, washed twice, dripped on multistrip slides (Highveld Biologicals, Transvaal, South Africa), air-dried over 4–6 hours, and frozen to −80°C for immunophenotyping or employed for cytological determination after staining with Romanowsky dyes [37] and non-specific esterase.
Blast colony assay
Blast colonies were grown following a previously described method [31] with some modifications. Magnetically selected 1×10⁴ CD34+ cells obtained from controls or patients were layered in duplicate in cross-culture experiments on preformed normal or aplastic SL for 2 hours to ensure optimal adherence; the unattached cells were then removed by standard washing with α-MEM. Progenitors affixed to SL were covered with 1 mL of 0.3% agar (final concentration) in α-MEM containing 30% FCS and cultured for 5 days at 37°C in 5% CO₂. Aggregates containing more than 20 cells were counted with an inverted microscope on days 5 and 7 of culture. Layers that had not been seeded with CD34+ cells served as negative controls. CD34+ cells from six normal individuals were also co-cultured on normal stroma and CFU-BI were scored to provide comparative values for colony numbers obtained from control progenitors layered on aplastic stroma.
Nonadherent CD34+ progenitors that had been washed off the stroma were pooled and cultured in the CPU-C assay [42]. As contents of decanted nonadherent progenitors were contaminated with stromal macrophages, aggregates of these cells were not scored; colonies of granulocytes and granulocyte-macrophage CFU containing more than 40 cells were counted, and the results of colony numbers from duplicates were divided by 2 and expressed per 10⁴ cells.

Immunofluorescence studies
Frozen multwell slides of LDBMC and selected cells from normal individuals or patients with aplasia were thawed and dried overnight, fixed in 100% acetone for 5 minutes, washed with PBS, and labeled for 45 minutes with FITC-conjugated CD3 (0.005 mL UCHT-1; Dako, Denmark) or CD19 (0.005 mL Dako HD-37) antibodies or CD14 (0.01 mL CD14, Mo2; Coulter) and CD34 (0.01 mL Anti-HPCA, My 10; Becton Dickinson) for indirect studies. With each case, positive [43] and negative (monoclonal antibody against β-galactosidase; Professor E.B. Dowdle, Department of Clinical Science and Immunology, University of Cape Town) controls were always included. Where relevant (in wells containing CD14 and CD34), a fluorescein-conjugated second rabbit antibody (Dakopatts) was added for 30 minutes. All excess reagents were washed off with PBS and the nuclei counterstained with 1 μg/mL ethidium bromide for 1 minute, washed, mounted, and read under ultraviolet illumination at 50 nm with a mercury gas lamp on a Nikon microscope.

Statistical analysis
Student’s t-test was used to assess the standard error of the difference between two means in the analysis of the blast colony assay data.

Results

Progenitor cell selection
Immunofluorescence studies on unselected LDBMC from patients with aplasia resulted in a median of 0.65% CD34+ (SD 0.39%) cells, significantly fewer progenitors than normal controls at 1.62% (SD 1.40%) (p = 0.002). Although the unselected population was not studied, results derived from the Initial number of CD34+ cells and selection recoveries suggests that the median efficiency of the method was 67.6% (SD 11.07) and 62.9% (SD 9.22) of the mononuclear cell population, respectively. Upon morphologic or immunophenotypic examination of the selected fraction, there were no obvious differences in the cell content.
Morphologically, undifferentiated blasts represented a median of 81 and 87% in each group. The majority of contaminating cells were of early granulocyte series, while monocytic cells were always <2% in each selected group. Immunofluorescence studies for the CD34+ antigen showed that 80% (SD 4.97) and 82.5% (SD 5.79) were reactive.

Stromal layers
Both normal and aplastic stroma became confluent at a median of 4 weeks (3–4 vs. 3–5). In one patient, stroma did not reach confluence by 5.5 weeks, when it started to detach. When inspected with an inverted microscope employing phase contrast, there were no major morphological abnormalities in the stroma from patients with narrow aplasia; their appearance was similar to that of normals. In adherent layers from four patients with aplastic anemia, fat cells appeared to be decreased and were not detected in some of the dishes in two patients and one control.

Cytochemical staining of the stroma
There was no difference in stromal appearance with the various dyes used in situ. Differential counts of stained cytopsin preparations from five normal controls (including two not in this study) showed that Sudan black positivity was present in a mean of 13% (SD 7.2) of cells, Oil Red O in 9% (SD 6.1), acid phosphatase 61% (SD 17.2), and alkaline phosphatase 72% (SD 19.5). The corresponding figures for the 16 aplastic patients were 16% (SD 7.7), 3% (SD 2.5), 55.5% (SD 11.4), and 64.3% (SD 20). None of these values differed significantly.

Blast colony assay
In seven normal controls, the mean CFU-BI number from cells selected for the CD34+ antigen and cultured for 5 days on aplastic stroma was 103.1 colonies/10⁵ cells (median 104, SD 30.4) (Fig. 1). Two types of colonies were obvious; the majority belonged to compact aggregates, which were well circumscribed, round, or more often elongated, following the body of fibroblast cells in the stroma, while the second group appeared to detach from the stroma and rise into the agar. The mean number of CFU-BI from selected progenitors from six normal individuals cultured on control stroma was 117 colonies/10⁵ cells (median 109, SD 20.4) (p > 0.05) on day 5; this number did not change significantly on day 7, when some clones became looser and started detaching from the adherent layer.
When aplastic CD34+ precursors were panned on normal stromal cultures, a mean of 41.06 colonies/10⁵ cells (median 29.5, SD 42.9) were scored, which was significantly lower than the colony numbers obtained from normal CFU-BI (mean 103.1, SD 30.4) on aplastic SL (p = 0.0002, exact two-tailed median test). The majority of the colonies were small and loose, with many scattered cells when observed on day 7. Although the colony number score did not change significantly, some aggregates continued to increase in size until day 10, when all cultures were terminated.
Fig. 1. Clonogenic potential of cells from patients with aplastic anemia. CD34" progenitors from patients with aplasia (n=15) were incubated on normal stromal layers and compared to normal precursors (n=7) grown on aplastic stroma (n=16) or control stromal layers (n=6). Results are expressed as stroma adherent CFU-BI colonies and stroma nonadherent fractions cultured on the CFU-GM assay.

None of the SI that were not seeded with selected progenitors showed any CFU-BI formation.

Stroma nonadherent clonogenic growth

When cultures from stroma nonadherent CD34" cells, decanted after 2-hour incubation and grown for 14 days in the presence of phytohemagglutinin-leukocyte conditioned medium (PHA-LCM), were scored for clonogenic growth and compared to production of CFU-BI, results revealed that both normal and aplastic CD34" had proportionately similar numbers of stroma adherent and nonadherent precursors. Specifically, when aplastic progenitors were incubated on normal stroma, the mean CFU-BI:CFU-GM ratio was 41.06 (SD 42.9)/142.6 (SD 104.8); the corresponding result from normal CD34" on aplastic stroma for CFU-Bi:CFU-GM was 103.1 (SD 30.4)/361.7 (SD 91.3) and similar to control progenitors off normal adherent layers at 117 (SD 20.4)/335 (SD 56.8) (p = 0.0009). Furthermore, when results of adherent and nonadherent aplastic colonies were combined as a total clonogenic potential and compared to the normal composite, diseased CD34" cells had significantly lower colony output (combined mean 185.8 vs. 447.1; p = 0.0009, exact two-tailed test).

These data indicate that both stroma adherent and nonadherent progenitors were decreased in aplasia and that alterations in the adhesion to the stroma were not a significant cause for reduction in the CFU-BI in this disease.

Discussion

In aplastic anemia, an overall activation of the immune system has been described, with increased bone marrow suppressor cell activity from CD8" Fcy' lymphocytes [25], in vitro elevation in interferon-γ, neopterin, and tumor necrosis factor (TNF) [19,44] levels by the bone marrow cells, and enhanced spontaneous secretion of these cytokines.

A few studies have suggested that the mechanisms of the pancytopenia originate from abnormalities in the marrow microenvironment [17,18,26,27,30,44], with marrow-suppressive effects and specific alterations in certain cell populations being demonstrable, both in the acute presentation and following immunosuppressive therapy [19,25,45]. When tested, however, these stromal cells secrete adequate amounts of G-CSF, GM-CSF, and IL-6 constitutively and after induction with IL-1 [46]. These observations are consistent with two recent studies [15,16] that suggest that defects at this level are rare in this disease.

Our initial work demonstrated that the stroma marrow aplasia adequately supports clonogenic progenitors [34]. Because of the marked reduction in progenitor cells obtainable from untreated patients, however, it was felt that with currently available methodology, extensive investigations into this population would be unsuccessful. Accordingly, subsequent work was directed into studying the clonogenic defect in the marrow that restores hematopoiesis after ALG therapy. To examine the interactions between the progenitors and the adherent layers, we have employed a CD34"-rich population using a short-term blast colony assay [31–33], since this provides a sharp endpoint in the form of quantifiable CFU-BI. Additionally, the adhesive interactions between the CD34" and the stroma could also be tested by analyzing, in clonogenic assays, the selected stroma nonadherent population and determining its proportions.

For this analysis, myeloid progenitors were obtained from the sternum of controls and the iliac crest of aplastic patients. Although variations in the cell populations obtained from different origins may occur, stromal cultures revealed no significant distinctions; clonogenic assays were based on selected precursors with purities and efficiencies that were also similar for both groups. Thus the source from which the material was obtained is not considered to affect these results.

In this study, the percentages of nonhematopoietic cells, such as endothelium and fibroblasts or B lymphocytes in the CD34"-selected population, were not determined [47]. However, these nonmyeloid cells from normal suspensions did not appear to affect the behavior of the aplastic stroma, as demonstrated by the comparable growth of control CFU-BI on normal or aplastic SI. We were unable to rule out the possibility that such populations present in the aplastic suspension could have had inhibitory effects on their own myeloid precursors, but this was thought improbable due to the low cell numbers remaining adherent to the stroma.

The original method was modified in that the irradiation of the allogeneic stroma was omitted to avoid damage to the possible presence of radiosensitive cells of the immune system that in vivo may modulate the aplastic hematopoiesis and to avert the release of burst growth factors that follows this physical insult [3,48].

Upon confluence, in situ morphological inspection of the cultures failed to demonstrate significant differences between normal and diseased stroma. Cytochemistry of the trypsinized and fixed SI showed similarity in the cell populations when
macrophages were stained with Sudan Black, fat cells with Oil Red O, and fibroblasts as well as reticulum cells with acid or alkaline phosphatase.

In the current analysis, we confirm that the myeloid stroma in aplastic anemia sustains normal hematopoiesis appropriately, with a clonogenic output that is no different from that of control layers. These data suggest that the prevailing levels of negative regulators are not functionally higher than those of control layers, since normal CFU-GM formation was well supported.

Of greater relevance is that in this disease, selected CD34+ precursors formed significantly fewer colonies on normal stroma, and the colonies generally showed suboptimal development. Furthermore, when nonadherent CD34+ cells were cultured in semisolid systems supplemented with PHA-LCM, CFU-GM colonies were proportionately similar but significantly lower in the disease group than in the controls (Fig. 1). These observations provide additional evidence that the fraction of dividing cells among the adherent and nonadherent populations is markedly reduced in this disease and that the decreased CFU-GM is not related to alterations in the adhesiveness to the stroma. These results are consistent with the hypothesis that the predominant hematopoietic defect lies within the progenitors. Whether similar mechanisms are operative during the acute presentation or this abnormality is only characteristic of the escape population that survives the initial aggression, remains unclear.

In an attempt to clarify this issue, we have studied these patients further in clonogenic assays, employing marrow fractions highly enriched for the CD34+ antigen and exposed to various combinations of growth factors belonging to the G protein and tyrosine kinase receptor superfamilies. Analysis of the data suggests that the derangement resides in the stem cells, at either the receptor or signal transduction level (unpublished observations).

We therefore conclude that the clonogenic defect of the marrow that restores hematopoiesis following ALG immunosuppression resides in the stem cell, while the levels of negative regulators in the stroma are not functionally higher than in normal marrow.

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In aplastic anemia progenitor cells have a reduced sensitivity to the effects of growth factors


Abstract: We have recently shown that in patients with aplastic anemia (AA) recovering following immunosuppressive therapy, the persistent reduction in the bone marrow clonogenic potential is unrelated to suppressive effects of the myeloid stroma and intrinsic to the hematopoietic progenitors. We examined the mechanisms of this defect by determining the response of the aplastic CD34+ clonogenic precursors to proliferative signals induced by hematopoietic growth factors and comparing their results with those of a control population. Light density bone marrow mononuclear cells were lymphocyte and monocyte depleted and enhanced for the CD34+ progenitors by immunomagnetic selection. Selected progenitors were then cultured in the mixed colony assay with incremental concentrations of combinations containing erythropoietin (Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and c-kit ligand. Bone marrow from aplastic patients had significantly fewer light density cells displaying the CD34 antigen (mean 0.63%, SD 0.35 vs. 1.62%, SD 1.4; p = 0.002). Dose response studies on aplastic CD34+ cells demonstrated that at low concentrations of Epo, IL-3 and GM-CSF, clonogenic growth was significantly impaired but achieved normal values at concentrations giving plateau growth in control cultures. However, for all colony types, responses to effective concentrations of c-kit ligand corresponded with those of controls. These data suggest abnormalities at the receptor or signal transduction levels.

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Key words: aplastic anemia; immunosuppressive therapy; hematopoietic progenitor cells; bone marrow failure; cytokines

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Despite significant advances in the therapy for aplastic anemia, little is known about the causes of bone marrow failure. While evidence suggesting primary stem cell abnormalities accumulates (1, 2), the inhibitory roles of the immune system and the bone marrow environment remain controversial (3, 4). To clarify these interacting mechanisms we had previously employed the blastoid colony forming units (CFU-BL) assay (4, 5) and found that, in patients who recovered after immunosuppressive therapy, the bone marrow stroma supports normal CFU-BL adequately (2, 6–8). This evidence confirmed results previously obtained with the long-term bone marrow culture system (LTBMC) (9, 10). It also indicated that in the aplastic microenvironment, the level of negative regulating peptides did not exceed that of control stroma. However, none of these studies provided further evidence regarding the possible mechanisms for bone marrow dysfunction.

Clinical experience indicates that some patients with aplastic anemia (AA) respond to therapy with pharmacological doses of erythropoietin (Epo) (11), granulocyte-macrophage colony-stimulating factor (GM-CSF) (12) or granulocyte-colony stimulating factor (G-CSF) (13). This suggests that, despite intense pancytopenia, under appropriate stimulation, amplification of the terminal elements is still
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possible, with consequent clinically significant improvements in blood values. These, however, are transient and only directed at the cell line under stimulation.

Studying the pathological lesion further, we exposed to combinations of growth factors, progenitors enriched for the CD34 antigen from patients with aplasia in partial or complete response to immunosuppressive therapy. Results of these studies demonstrate that the proliferative potential in these precursors is retained, but a significantly reduced sensitivity in these cells to the tested combination of peptides causes the reduced clonogenic response.

Materials and methods

In a pilot investigation patients with acute aplasia or subjects with this disease who were recovering after immunosuppressive therapy were studied and their results compared to a control population (8). In that study no CD34+ progenitor cells were detectable in the untreated group, so the current analysis was restricted to only those patients who had responded to anti-lymphocyte globulin therapy.

The present cohort has been comprehensively described elsewhere (6, 8, 14). Briefly, we investigated 15 subjects of median age 30 (range 18–61) presenting with acute aplasia (15) who had responded to anti-lymphocyte globulin (ALG), or, in the case of three, to additional cyclosporine immunosuppression. They were studied at a median of 4 yr from the initial treatment (range 1–8). Another patient in partial recovery, after therapeutic abortion for hypoplasia, was also tested. Their hematological data are presented in Table 1. Morphologically, variable degrees of dysplasia were evident in the blood and the bone marrow in all but one patient. Two developed a positive Ham’s test and clinical features of paroxysmal nocturnal hemoglobinuria at 2 and 4 yr after presentation.

Patients’ bone marrow was aspirated from the posterior iliac crest under 1M pethidine hydrochloride (1 mg/kg) analgesia, given 30 min before the local anesthesia. Seven hematologically normal individuals undergoing sternotomy for cardiac surgery under general anesthesia comprised the control population. While the anatomical source of marrow of the two populations differed, possible variations in the frequency of myeloid precursors were not considered likely to affect the in vitro culture results significantly, as only progenitors enriched for the CD34+ membrane antigen were employed for these clonogenic assays. All consented to donate bone marrow according to institutional guidelines of the University of Cape Town and Groote Schuur Hospital.

Selection of the hematopoietic progenitor cells

Methods employed for purifying and selecting the progenitor cell population have been described in detail elsewhere (8). Briefly, control or aplastic mononuclear cells were recovered from a density gradient (1.077 g/mL, Histopaque, Sigma, USA), monocyte and lymphocyte depleted by 2 h adherence on plastic dishes and incubation with CAMPATH 1 M and complement (16). The resulting monocyte- and lymphocyte-poor concentrates were labelled at 4 °C with 0.02 mL/10⁷ cells of anti-human CD34 antigen murine monoclonal antibody (Anti HPMA-1, My 10, Becton and Dickinson, Sunnyvale, California). Labelled cells were washed and exposed at 4 °C to paramagnetic beads covalently bound to affinity purified goat IgG (M-450, product no. 125.01, Dynal, Oslo, Norway) (17). This target population was then harvested with a magnet applied to the sides of the tube (MPC-1, Dynal, Oslo, Norway). Cells were freed from beads by re-suspension in 0.1 mL of phosphate buffered saline (PBS) containing 0.01 mL of a polyclonal murine immunoglobulin (Detchahead, Dynal, Oslo, Norway) (18). Their viability was determined, cells were counted in a hematocytometer and their numbers were adjusted to a concentration of (1–5) × 10⁶/mL.

Aliquots of cells obtained from the initial density gradient and from the final separation were incubated overnight at 37 °C in medium containing 15% foetal calf serum (FCS). They were washed twice, dripped onto multitest slides (Highveld Biologicals, Transvaal, South Africa), air-dried over 4–6 h and frozen to −80 °C for immunophenotyping.

Hematopoietic growth factors

Recombinant human (rh) cytokines (Amgen Thousand Oaks, California, USA); rh-IL 3 (specific activity of 10⁷ U/mg), rh GM-CSF and c-kit ligand (specific activity 1 x10⁶ U/mg each), at protein concentrations of 0.5, 0.5 and 2 mg/mL, respectively, were diluted to 10² ng/mL and stored at 4 °C.
Poor sensitivity to growth factors of progenitor cells in aplastic anemia

Epo was donated by Bioclones (Sandton, Transvaal), and once reconstituted, was kept at 4°C at a concentration of 1000 U/mL.

Clonogenic assays

Normal or aplastic selected CD34 progenitors were cultured in vitro for 14 d at 37°C, at a cell density of (1–5) x 10³ in Iscove's Modified Dulbecco's Medium (Gibco BRL, Life Technologies, Paisley, Scotland) (IMDM), supplemented with l-glutamine, 30% FCS, 5 x 10⁻⁵ M 2-mercaptoethanol, 0.3% agar (final concentration) and incremental concentrations of growth factors. The selected cytokines were four concentrations of Epo in the presence of IL-3 10 ng/mL; increments of IL-3 with Epo 2 U/mL, of GM-CSF in combination with Epo 2 U/mL and of c-kit ligand in association with Epo 2 U/mL and IL-3 10 ng/mL. Dose responses were constructed based on a pilot study in normal individuals, where starting growth factor concentrations represented 25–50% of maximal clonal efficiency. The highest dose usually expressed concentrations well above plateau growth values for the cytokines tested. All cultures were performed in duplicate and their averages then subjected to statistical analysis.

Cultures were fixed in 10% glutaraldehyde and stained with May-Grünwald-Giemsa for permanent recording. Granulocyte-macrophage colony forming units (CFU-GM) were defined as myeloid aggregates (granulocytic, monocytic-macrophage or mixed) containing more than 40 cells. Erythroid colony forming units (CFU-E) were described as erythroid clusters of 4–99 cells, while burst forming units-erythroid (BFU-E) were expressed as colonies containing more than 100 cells or aggregates of 3 or more subclusters.

Immunofluorescence studies

Frozen multiwell slides of light density and selected cells from normal subjects or patients with aplasia were thawed and air dried overnight, fixed in 100% acetone, and labelled with CD34 (0.01 mL Anti-HPCA, My 10, Becton Dickson, USA) for indirect studies. A fluoresceine conjugated second rabbit antibody (Dakopatts) was then added. Positive (19) and negative (monoclonal antibody against beta galactosidase, Professor E. B. Dowdle, Department of Clinical Sciences, University of Cape Town) controls were always included. Slides were mounted and read under ultraviolet illumination at 50 nm with a mercury gas lamp.

Statistical analysis

Raw values of colony scores were subjected to logarithmic transformation with the formula \( Y' = \ln(Y + 1) \) because of the marked dispersion of the data. Percent increment (%) in colony numbers of 4 growth factor combinations were compared by the formula \( \%Z = (Y'X/100) - 100, \) where \( Y \) represented the colony number under investigation and \( X \) the colony values obtained with the combination IL-3 10 ng/mL+Epo 2 U/mL. Means, standard deviations and confidence intervals were calculated and a two-way analysis of variance was employed to determine the significance of differences between groups of cultures or a single culture versus a control group. Multivariate analysis was performed to define correlation between sets of observations, discriminant functions and clustering of data (20).

Results

Progenitor cell selection

Immunofluorescence studies on unselected light density cells from patients with aplasia resulted in a median of 0.65% CD34+ (SD 0.39) cells, which were significantly fewer than in normal controls at 1.62% (SD 1.40) (two way t-test \( p=0.002 \)). Although immunofluorescence studies were not performed on the unselected population, the efficiency of the procedure was calculated from the initial CD34 reacting population within the mononuclear cell fraction and the cells obtained after immunomagnetic selection, resulting in 62% recovery.

Morphological and immunophenotypic examination of the selected fraction revealed no obvious difference in the cell content. Morphologically, undifferentiated blasts represented a median of 81 and 87% in each group. Immunofluorescence studies for the CD34 antigen showed that 80% (SD 4.97) and 82.5% (SD 5.79), respectively, were reactive, while monocytic cells were always <2%.

Erythropoietin (Epo) (Fig. 1)

Dose response with incremental concentrations of Epo at 0.2, 2, 10 and 100 U/mL in the presence of IL-3 10 ng/mL showed increased numbers of BFU-E in both patients and controls (Table 2). In the normal group the maximal clonogenic efficiency for erythroid bursts was reached at 10 U/mL. Optimal CFU-E growth was already achieved at the lowest concentration, while no significant effects were induced on myeloid clones by erythropoietin at any dose.

In the aplastic group, however, compared to normal CD 34+ cells, BFU-E numbers were
ERIOTROPOPETIN

GM-CSF

BFU-E

CFU-E

CFU-GM

Fig. 1. Comparative results in the clonogenic growth between patients with aplastic anemia and normal individuals. Values in the vertical axis are expressed as results of mean colony numbers from $5 \times 10^5$ normal and aplastic CD34+ progenitors while those in the horizontal axis denote the concentration of the growth factor under investigation. Dose response to Epo 0.2, 2, 10 and 100 U/mL in the presence of IL-3 10 ng/mL and GM-CSF 0.1, 1, 10 and 50 ng/mL in the presence of Epo 2 U/mL are shown. Results are expressed as mean of $Y = \ln(Y + 1)$. With Epo, differences at 0.2 and 2 and 10 U/mL were significant for BFU-E colonies ($p < 0.05$). With GM-SCF differences at 0.1 and 1 ng/mL were significant for BFU-E and CFU-GM colonies ($p < 0.05$).

significantly lower at 0.2, 2 and 10 U/mL ($p < 0.001$, 0.001 and 0.05). Erythroid burst numbers from the patients continued improving significantly even at 100 U/mL (0.2 and 2 U/mL vs. 100 U/mL; $p = 0.01$), approaching control values, without achieving plateau growth, in contrast to the control group. The CFU-E numbers at all concentrations were less numerous in the patients than in normal samples but this difference in the results did not achieve significance ($p > 0.05$). CFU-GM were unaffected by changes in Epo concentration.

Granulocyte-macrophage colony stimulating factor (Fig. 1)

Control and aplastic progenitors were cultured with Epo 2 U/mL and GM-CSF at concentrations of 0.1, 1, 10 and 50 ng/mL. Erythroid bursts from normal marrow peaked at the lowest concentration and appeared inhibitory at higher doses, while CFU-E numbers were not affected by higher growth factor concentrations. Significant improvements in CFU-GM followed increments in the concentrations of this cytokine between 0.1 and 50 ng/mL ($p = 0.04$).

In the patients with aplasia, the numerical increment in BFU-E was significant from the lowest concentration of GM-CSF to 10 ($p < 0.05$) and 50 ng/mL ($p < 0.01$). Compared to the control population, BFU-E numbers were significantly lower than those of normal cultures at 0.1 and 1 ng/mL ($p < 0.001$ and 0.001). For CFU-E, GM-CSF was already at saturating concentrations at the lowest dose and no further improvement in colonies was experienced. When the concentrations were increased from 0.1 to 50 ng/mL, GM-CSF significantly stimulated the proliferation of aplastic myeloid colonies ($p = 0.01$). Compared to control values, at concentrations of 0.1 and 1 ng/mL, CFU-GM numbers were significantly lower ($p < 0.023$ and 0.01) but colony scores continued rising when supplemented with 50 ng/mL, at this concentration approaching control values.

Interleukin 3 (IL-3) (Fig. 2)

Bone marrow selected cells were cultured with Epo 2 U/mL and IL-3 at 1, 10 and 50 ng/mL. In the normal precursors IL-3 also gave a maximal clonal expansion at 10 ng/mL for all colony types, while at 50 ng/mL some inhibitory action on the erythroid colonies was noted.

In the aplastic group, increments in the numbers of all colony types were again observed. BFU-E numbers were significantly higher in cultures containing 50 ng/mL than in the lowest concentration ($p < 0.05$). Compared to control values, colony numbers from aplastic clones were significantly lower for BFU-E at 1 ng/mL ($p = 0.001$) and at 10 ng/mL ($p = 0.001$) but matched normal samples at the top concentration. CFU-E numbers were not significantly changed by increments in IL-3. CFU-GM proliferation was well preserved, showing elevation in colony numbers matching the results obtained by the control progenitors (Fig. 2).

Stem cell factor (c-kit) (Fig. 2)

Normal and aplastic progenitors were cultured with Epo 2 U, IL-3 10 ng/mL and stem cell factor at concentrations of 1, 20 and 200 ng/mL. In the normal assays, the addition of incremental concentrations of c-kit ligand to the basic IL-3 + Epo combination showed insignificant improvements in the BFU-E and CFU-GM numbers, while CFU-E development was not affected.
Poor sensitivity to growth factors of progenitor cells in aplastic anemia

Table 2. Colony assays

<table>
<thead>
<tr>
<th>Cytokine combination</th>
<th>Type of colony</th>
<th>BFU-E</th>
<th>Control</th>
<th>CFU-E</th>
<th>Control</th>
<th>CFU-GM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aplastic</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>IL-3 10 ng + erythropoietin Epo:</td>
<td>0.2 U/mL</td>
<td>n = 11</td>
<td>0.81</td>
<td>0.95</td>
<td>2.90</td>
<td>0.89</td>
<td>2.95</td>
</tr>
<tr>
<td></td>
<td>2 U/mL</td>
<td>n = 15</td>
<td>2.10</td>
<td>1.32</td>
<td>3.97</td>
<td>0.54</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>10 U/mL</td>
<td>n = 15</td>
<td>3.03</td>
<td>1.16</td>
<td>4.14</td>
<td>0.49</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>100 U/mL</td>
<td>n = 15</td>
<td>3.57</td>
<td>0.71</td>
<td>3.36</td>
<td>0.54</td>
<td>3.34</td>
</tr>
<tr>
<td>Epo 2 U/mL + GM-CSF GM-CSF:</td>
<td>0.1 ng/mL</td>
<td>n = 7</td>
<td>1.62</td>
<td>0.95</td>
<td>3.66</td>
<td>0.71</td>
<td>3.23</td>
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<tr>
<td></td>
<td>1 ng/mL</td>
<td>n = 15</td>
<td>1.67</td>
<td>1.27</td>
<td>3.37</td>
<td>0.49</td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>10 ng/mL</td>
<td>n = 15</td>
<td>2.53</td>
<td>0.99</td>
<td>3.04</td>
<td>1.09</td>
<td>2.94</td>
</tr>
<tr>
<td></td>
<td>50 ng/mL</td>
<td>n = 15</td>
<td>2.98</td>
<td>0.70</td>
<td>3.57</td>
<td>0.82</td>
<td>3.23</td>
</tr>
<tr>
<td>Epo 2 U/mL + Interleukin 3 IL-3:</td>
<td>0.1 ng/mL</td>
<td>n = 15</td>
<td>1.50</td>
<td>1.08</td>
<td>3.26</td>
<td>0.76</td>
<td>3.26</td>
</tr>
<tr>
<td></td>
<td>10 ng/mL</td>
<td>n = 15</td>
<td>2.10</td>
<td>1.32</td>
<td>3.87</td>
<td>0.54</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>50 ng/mL</td>
<td>n = 15</td>
<td>2.94</td>
<td>0.98</td>
<td>3.02</td>
<td>0.96</td>
<td>3.19</td>
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<tr>
<td>Epo 2 U/mL + IL-3 10 ng/mL + stem cell factor SCF:</td>
<td>0.1 ng/mL</td>
<td>n = 7</td>
<td>1.76</td>
<td>1.69</td>
<td>4.11</td>
<td>0.42</td>
<td>2.61</td>
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<tr>
<td></td>
<td>20 ng/mL</td>
<td>n = 7</td>
<td>3.05</td>
<td>1.01</td>
<td>4.57</td>
<td>0.54</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>200 ng/mL</td>
<td>n = 7</td>
<td>3.99</td>
<td>0.50</td>
<td>4.26</td>
<td>0.71</td>
<td>2.66</td>
</tr>
</tbody>
</table>

Values from individuals with aplasia were significantly lower than those from the control group only for BFU-E at 1 ng/mL (p < 0.05), as at this concentration the prevalent effects were provided by the basic IL-3 + Epo combination (21). At effective c-kit ligand concentrations (≥20 ng/mL), results improved to values not significantly different from those of control cultures (Table 2 and Figs. 2 and 3). CFU-E numbers were not altered by changes in SCF concentration. CFU-GM numbers also improved but did not differ significantly at the three concentrations or from control values.

The effect of saturating concentrations of IL-3, GM-CSF, Epo and stem cell factor on the basic IL-3 10 ng/mL and Epo 2U/mL were studied by determining the percent increment of absolute colony numbers in each group. As Fig. 3 shows, with all combinations, aplastic precursor cells had a proportionally higher increment in colony numbers. This was most significant for the combination containing maximal concentrations of c-kit ligand leading to enhancements in CFU-GM and BFU-E by 330% and 263% (p < 0.05 and 0.01), for these colonies (Fig. 3).

In summary, results of dose responses in the mixed colony assay showed that compared to those of the normal population, aplastic CD34 + progenitors displayed a significantly reduced sensitivity to Epo, GM-CSF and IL-3. By contrast, exposure of aplastic precursors to effective concentrations of c-kit ligand (≥20 ng/mL) led to significant BFU-E and CFU-GM enhancement, superior to saturating concentrations of any other growth factor. Resultant increments were proportionally higher than those of normal cultures (Fig. 3), approaching values not significantly different from those of normal precursors.

Multivariate analysis segregated the results for all three-colony types of cultures supplemented with c-kit ligand at biologically effective concentrations (≥20 ng/mL) from the remaining studies. Interpretation of these data would suggest that cultures containing c-kit ligand were affected by independent interactions from those induced by the other cytokine combinations.

Discussion

We have previously shown (6) and confirmed (8) that the bone marrow stroma in aplasia supported hematopoiesis adequately, suggesting that the primary defect lay within the hematopoietic stem cell (7, 8). The objectives of the current investigation were to determine in dose response studies, the proliferative state of selected aplastic CD34 + progenitors greatly depleted of accessory cells.
Motivations for the choice of growth factors for the study were four-fold. Firstly, some of these cytokines had been shown to be clinically useful in vivo (11–13, 22). Secondly, these combinations had already been tested in studies containing unfractionated marrow cells (22–25), furthermore they included peptides belonging to both the cytokine and tyrosine kinase receptor super-families (26–30). Lastly, these culture combinations included early and late acting cytokines with pleiotropic functions that included erythroid burst promoting activity such as IL-3 and GM-CSF and stem cell factor (21, 26, 28).
Poor sensitivity to growth factors of progenitor cells in aplastic anemia

induce the release of TNF (27), or other negative regulators, limiting the proliferative effects of growth factors on the hematopoietic progenitors.

These experiments have also documented that, despite a sub-optimal response to the more restricted stimulators (Epo 2 U/mL and IL-3 10 ng/mL), adding effective concentrations of c-kit ligand significantly corrected the inferior colony development. c-kit ligand has little clonogenic activity on its own (21), but at appropriate concentrations it was synergistic with other cytokines. Furthermore, in aplastic progenitors, the proportional increment (% change) in the colony numbers of cultures containing this cytokine was superior to that of control studies. It exceeded the basic IL-3 10 ng/mL + Epo 2 U/mL by 563.3% for erythroid and myeloid colonies together, leading to the highest scores for BFU-E and CFU-GM (Fig. 3). This resulted in clonogenic yields not significantly different from those of control cultures, supporting the view that in this disease the clonal potential remains preserved. Multivariate analysis revealed a segregation of cultures supplemented with effective concentrations of c-kit ligand, from those grown with other GF combinations, suggesting independent interactions affecting the cultures containing this growth factor. In this context, other investigators had reported similar results (24, 25, 33).

We have previously shown, with the CFU-bl assay, that cells in the aplastic stroma do not exert excessive inhibition on control CD34+ progenitors (7, 14). In the current analysis, we have documented that appropriate response to certain growth factors are observed only at high growth factor concentration or in the presence of c-kit ligand, suggesting alterations in the signaling pathways for cell division. Reduced response to growth factors may result from abnormalities in the receptor densities or affinities for the ligand, leading to sub-optimal clonogenic growth. Alternatively, IL-1 or IFN, which enhance Fas receptor expression, may inhibit colony formation through induction of apoptosis (34, 35).

However, in this study, the described derangement in the responses to GF ranged widely over three cytokines, indicating alterations in shared structures or in pathways signaling for cell division. Since cooperation and integration of signals between pathways are important in the mitogenic response (30), supplementation of cultures with c-kit ligand may lead to correction of the signals relayed and to correction of the cellular proliferation. This may have important therapeutic implications.

It is concluded that in patients with aplastic anemia treated with ALG in combination with HDMP, intrinsic abnormalities in the CD34+ population result in subnormal clonogenic growth. Whether during the initial presentation, a more profound abnormality of the same pathways leads to the severe pancytopenia, or whether this derangement is a characteristic of the escape population surviving the original attack, remains to be determined.

Acknowledgements

Our gratitude is extended to Professor L. Wilson for extensive discussion; and to Dr. S Iuscu for the comprehensive statistical analysis.

This work was supported by the University of Cape Town Leukaemia Centre and Staff Research (Foote, Becker and Cancer) Fund, the Gwendoline Moore Trust, the National Cancer Association, the Medical Research Council, and the Michael Chanani, Kaliski and M.A. Richardson Bequests.

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Novitzky & Jacobs


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bone marrow aspiration revealed a hypercellular bone marrow with M:E 25:1 and marked increase in granulocytopoiesis. The differential count was: neutrophils 12%, blasts 1%, metamyelocytes 13%, myelocytes 51%, promyelocytes 14%, basophils + mast cells 10%, erythropoiesis was mildly megaloblastic without any features of dyserythropoiesis. A severe degree of dysgranulopoiesis was present. There were many abnormal promyelocytes and mononuclear promyelocytes, Pelger-Huet anomaly and neutrophils with ring nuclei were seen. Many blasts had Auer rods. An occasional megakaryocyte was seen. There was focal increase in plasma cells in the particles. There was a marked increase in tissue mast cells in the bone marrow. Mostly these cells were located in the particles, besides being present in clumps or in isolated form (Fig 1). Many of them were fusiform and other were oval or round. The cytoplasm contained numerous metachromatic granules, which covered the nucleus and the cytoplasm. The relatively small nuclei were mostly bi-lobed with an occasional mast cell having a single lobed nucleus. Most of them were loaded with granules, but some were deprived of granules or showed sparse granulation. Cytochemically these mast cells were strongly positive for toluidine blue and acid phosphatase, positive for Sudan black B, periodic acid-Schiff (PAS) and naphthol AS-D chloroacetate and negative for myeloperoxidase and leucocyte alkaline phosphatase. On the basis of these findings the diagnosis of MDS-RAEB-t with malignant mastocytosis was established. The patient was neither available for treatment nor follow-up.

Among several reports on the association of neoplastic haematological disease with mast cell disorders, the occurrence of MDS seems to have been missed till the observation by Travis et al (1988) in systemic mastocytosis. This might be due to the fact that MDS is generally accepted to cover a rather ill-defined group of disorders of haemopoietic stem cells (Dash et al., 1991). Although it is generally accepted that prognosis in these cases is determined by the associated haematological disease rather than mastocytosis, the cases of MDS reported by Travis et al. (1988) lead an aggressive course. Nine out of 10 died of which five (55-5%) had developed acute leukaemia. This incidence is much higher than that usually reported in MDS.

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REFERENCES


MARROW STEM CELL AND STROMA CELL FUNCTION IN APLASTIC ANAEMIA

Using a modification of the long-term bone marrow culture assay (Dexter et al., 1977) Marsh et al. (1991) have recently provided data that CD34 positive myeloid precursor cells derived from patients with aplastic anaemia have a significantly lower cloning efficiency than normal progenitors thereby suggesting an intrinsic defect in this population.

Preliminary data from a parallel and ongoing study in our department provides support for this observation. Thus, in an

Br J Haematol 1991; 79: 531-533
assay that exploits the adhesive properties of undifferentiated blasts colonies designated 
CDFUu (Gordon et al. 1987) we have examined the growth-promoting properties of stroma 
derived from both patients with aplastic anaemia and normal 
volunteers in addition to the cloning efficiency of their respective bone marrow stem cells in cross-culture 
experiments. Furthermore, we have measured the response of the same population, once depleted of accessory cells, to recombinant growth factors.

After informed consent had been obtained haematologically 
normal individuals undergoing thoracotomy, or su-
jects with aplastic anaemia, donated 30–40 ml of bone 
marrow which, following separation on ficoll-hypaque gra-
dient with a specific gravity of 1.077 g/ml the harvested light-
density cells were separated into two fractions.

Stromal cultures were established from normal and aplas-
tic subjects by suspending 5 x 10^6 cells in alpha minimum 
esential medium containing 12.5% horse serum, 12.5% 
feetal calf serum, 10^-4 M hydrocortisone and culturing these 
in 30 mm petri dishes at 37°C in 5% carbon dioxide and high 
humidity. Medium was changed weekly and upon confluence 
the layers were irradiated at 75 cGy/min to a total of 15 Gy 
(Fig 1).

The second fraction was monocyte depleted by 2 h 
incubation in 90 mm plastic petri dishes and 5 x 10^6 cells 
overlayered on the performed normal and aplastic stromal 
cultures for 2 h at 37°C for adhesion to take place. Cells not 
adherent to the stroma were removed by standardized 
washing with tissue culture medium and the presence of 
progenitors in the effluent medium quantitated in the 
GM-CFUc assay. Stromal adhesive precursors were imobi-
lized in 0-3% agar and cultured for 5 d at 37°C in a high 
carbon dioxide and humid atmosphere. CFUu were scored as 
colonies when aggregates contained more than 20 cells.

The remaining monocyte-poor marrow cells were incu-
bated with anti-CD34 (My 10 Becton Dickinson) at 4°C for 30 
min, excess antibody washed off and the labelled cells exposed to 
5–15 x 10^6 paramagnetic particles coated with high-
affinity goat anti-mouse immunoglobulin (Magnetic Insti-
tute, Boston, Mass.). A positive selection was achieved by a 
magnetic field applied to the sides of the tube.

Target populations were enumerated, characterized morphologically and by immunofluorescence, after which 
between 5 x 10^5 and 5 x 10^6 cells were cultured using a 
mixed colony assay in the presence of 2 units erythropoietin, 
50 μg/ml rh recombinant human interleukin 3 (II3) or 
granulocyte-macrophage colony stimulating factor (GM-
CSF), mercaptoethanol, 30% FCS in 0-3% agar. All results 
were corrected and expressed as colonies/10^5 cells.

To date, five normal controls, two patients with untreated 
aplastic anaemia (UA) and three with this disease in partial 
response following antilymphocyte globulin (TAA) have 
been studied. There was no morphological difference between 
normal stroma and that derived from patients with TAA 
whilst that from UA showed a marked reduction in macro-
phages.

Both normal and aplastic stromal layers supported the 
formation of CFUu to an equal extent. There was, however, a 
marked difference in the number of colonies generated from 
the marrow of patients with TAA when grown over normal 
stroma, in addition to which these were small and poorly 
defined. No clonogenic growth occurred from progenitors 
derived from patients with UA.

When the population of cells not adherent to stroma was 
cultured to GM-CFUc were present in the normals whereas 
16–40 colonies were formed in the TAA patients.

The CD34 positive population was quantitatively de-
creased in TAA. However, cultures of the accessory-poor cell 
fraction, defined as containing less than 2% CD3, CD19, 
CD14 positive cells, and positively selected to a purity greater than 
80% showed a marked reduction in the colony growth 
using the GEMM:CFU assay when compared to normal 
precursors in response to recombinant IL3 and GM-CSF 
(Fig 2).
As Dr Marsh and her colleagues point out, there is growing evidence of intrinsic abnormality in the haematopoietic progenitor cell function at least in some of the patients and this is reflected in our experience that included atypical blast colony formation over pre-formed stroma, decreased adhesiveness and impaired response to recombinant growth factors. It is therefore not surprising that the mature blood elements may be quantitatively abnormal in this disease (De Planque et al., 1989). Although our own data is still inconclusive, a hypothesis could be proposed that in some cases of aplastic anaemia the haematopoietic defect may be present before the onset of the pancytopenia which is the result of an immunologically mediated surveillance mechanism mounted to suppress the abnormal haematopoiesis. Biological immune response modulation, with antilymphocyte globulin, leads to an improvement in the peripheral blood count values as well as in marrow cellularity but is unlikely to correct the underlying defect.

ACKNOWLEDGMENTS

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REFERENCES


PROGNOSTIC VALUE OF CD34 EXPRESSION IN DE NOVO ACUTE MYELOBLASTIC LEUKAEMIA

In a recent paper by Geller et al. (1990) the expression of CD34 by leukaemic cells of patients with acute myeloblastic leukaemia (AML) was found to be significantly associated with resistance to remission induction therapy. Consequently, CD34-positive patients had a clearly lower complete remission (CR) rate. Cases expressing CD34 also had a much higher incidence of secondary AML (sAML) and a history of antecedent haematological disorder (AHD). As in the experience of other investigators (Borowitz et al., 1989), patients with sAML or AHD were also more likely to fail induction remission treatment. By using multivariate analysis and adjusting for other clinically relevant covariates, Geller et al showed that the prognostic value of CD34 expression tended to be independent (P=0.066).

In an attempt to further clarify the prognostic importance of CD34 expression in AML, we have analysed its possible relationship with leukemic resistance in a series of 27 patients with de novo AML. Response was assessed by a bone marrow aspirate performed on day 15 after chemotherapy and weekly thereafter. CR status was defined by standard criteria. Patients who died before response could be properly evaluated were considered ineligible for the purposes of this study.

Induction remission therapy consisted of one cycle of a conventional 3+7 regimen including daunorubicin 60 mg/m² or mitoxantrone 12 mg/m² and cytarabine (200 mg/m²/d). As in Geller’s study, CD34-positive cases were defined as those with more than 10% blast cells showing reactivity with CD34 monoclonal antibody (My10). The main clinical and haematological characteristics of patients according to CD34 expression are depicted in Table I. CR rate was clearly related to CD34 (P=0.058) and myeloperoxidase (MPO) reactivity (P=0.030). All 14 CD34-negative cases entered CR as did all 12 cases with more than 40% MPO reactivity.

Table I. Clinical and haematological characteristics according to CD34 reactivity

<table>
<thead>
<tr>
<th>CD34-positive</th>
<th>CD34-negative</th>
<th>P-value</th>
</tr>
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<tr>
<td>No.</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>Age (yr), median (range)</td>
<td>50 (18–67)</td>
<td>52 (32–68)</td>
</tr>
<tr>
<td>WBC (x 10⁹/l), median (range)</td>
<td>38 (2–3–113)</td>
<td>14 (1–191)</td>
</tr>
<tr>
<td>MPO reactivity (%), median (range)</td>
<td>3 (0–100)</td>
<td>53 (0–100)</td>
</tr>
<tr>
<td>FAB (no. of cases)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>1</td>
<td>1</td>
</tr>
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<td>M3</td>
<td>4</td>
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<td>M6</td>
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</tr>
<tr>
<td>M7</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>No. of CR (%)</td>
<td>7 (54%)</td>
<td>14 (100%)</td>
</tr>
</tbody>
</table>

MPO = myeloperoxidase; CR = complete remission; NS = not statistically significant.
SUMMARY

The in vitro culture of bone marrow is an established method for the study of normal and abnormal haematopoiesis. In a semi-solid agar system, marrow progenitor cells (CFU) of the granulocyte and macrophage lines will clone in the presence of specific colony-stimulating factors (CSF) and appear in the matrix as clusters (6-50 cells) or colonies (more than 50 cells). Growth is not normally obtained from peripheral blood unless this is first concentrated. To characterize the morphology of these cells in situ, a method has been developed in which the entire plate is fixed and mounted on a slide. After drying, this preparation is well suited to staining with a variety of biological dyes and furthermore provides a permanent and detailed record of the entire culture.

Analysis of 414 studies in a variety of clinical conditions forms the basis of this communication. In a 35-mm Petri dish normal marrow grows 30 colonies (SEM ± 2,49) and 30 clusters (SEM ± 3,59) at 12 days of culture resulting in a colony:cluster ratio of 1:1.3. The technique was found useful in our bone marrow transplantation programme where scanty growth in culture identified those donor grafts containing a paucity of stem cells, which is one explanation for the failure to obtain haematopoietic reconstitution. In preleukaemia only 2.76 colonies (SEM ± 0,85) and 15 clusters (SEM ± 4,24) were obtained.

In overt leukaemia, whether arising ab initio or following relapse, plating efficiency was similarly reduced to 5,2 colonies (SEM ± 1,34) although 64 clusters (SEM ± 12,54) were found resulting in a colony:cluster ratio between 1:5 and 1:12. With successful therapy this pattern returns to normal, but reappearance of disturbed growth or aberration of colony:cluster ratio signals a relapse or the presence of minimal residual disease, a pattern that may occur when peripheral blood and marrow are still morphologically normal. In the myeloproliferative syndrome CFU become demonstrable in the peripheral blood as the disease progresses, and metastasize to either the accelerated phase or to blastic transformation results in changes similar to those found in leukaemic marrow.

These studies illustrate the potential practical value of this technique. Further applications include the exploration of aetiological mechanisms in patients with neutropenia and bone marrow aplasia, or in elucidating humoral or cellular factors inhibiting growth in patients with tumours such as myeloma. In vitro culture systems have diagnostic potential in haematology, and further evaluation will define their role in current practice.


The mechanisms controlling the orderly proliferation and maturation of haematopoietic cells within the bone marrow are complex and they have proved difficult to analyse because, until recently, there was no practical way of examining the influence of the individual regulating factors. Liquid suspension cultures of marrow suffer from many of the same limitations as in vivo experiments in that relatively small segments of the whole process cannot be isolated for study. Appreciating the need for alternative analytical techniques, Pluznik and Sachs' and Bradley and Metcalf' extended the experience of virologists that transformed cells will grow in vitro by attempting to culture tumour cells using similar methods. The two groups tried independently to obtain clonal growth in agar using mouse leukaemia or AKR lymphoid leukaemia cells respectively, but both were initially unsuccessful. Further experiments were based on the technique described by Puck and Marcus' in which feeder layers of various cells were employed to obtain colony formation. However, although growth did occur, analysis of the cell aggregates showed that these were composed of neutrophilic granulocytes and macrophages derived from the underlayer and not of tumour origin. Nevertheless, these and other studies' established that a specific growth factor was necessary for colony formation and that this was supplied by cells from the underlayer.

Rapid progress has been made from these important initial observations. Thus, Pike and Robinson's successfully applied the double-layer technique to the study of human bone marrow, while Chervenick and Boggs' substituted methylcellulose for agar as the matrix to support cell growth. Subsequently, Axelrad et al.' demonstrated that erythroid colony formation could be obtained in the presence of erythropoietin. Metcalf et al.3,9 have been prominent in much of the development of semi-solid agar systems including isolation and purification of colony-stimulating factors' and the use of mitogen-stimulated lymphocytes to support the growth of eosinophils as well as megakaryocytes. Furthermore, it was observed that B and T lymphocytes and haematopoietic stromal cells could be successfully cultured in the system.
containing 2-mercapto-ethanol. These various achievements have been reviewed by Metcalfe and it is clear that the basic technique may, with modifications, provide a practical approach to the analysis of the various physiological processes that govern cell growth, including the relationship between haematopoietic cells and supporting stroma.

The same approach has been used to explore functional differences that may be recognizable between normal and neoplastic cells. In the case of leukaemia it has been reported that the tumour cells may be distinguished from their normal counterparts in culture, and this important observation is presently being further evaluated. The most obvious deviation that samples of leukaemic marrow show from normal are alterations in plating efficiency and disturbances in colony : cluster ratio. The latter is presumed to reflect, at least in part, profound suppression of normal haematopoietic populations. Other explanations are that leukaemic progenitor cells are inherently unable to mature normally, or that their response to regulatory factors may be defective. A variety of other malignancies have been examined by in vitro culture of bone marrow and most of this material has recently been analysed and reviewed by Metcalfe. It is evident that these methods provide a means for the examination of a host relationship with the neoplasm; a chance to define tumour kinetics and provide at least one way of testing cytotoxic regimens.

One of the major limitations of the agar culture system is the difficulty in defining morphological detail of constituent cells within the colonies or the clusters, and this difficulty is reflected in the variety of techniques used for their study. The latter extend from phase-contrast microscopy through a variety of supravital techniques to those which necessitate aspiration of the colony from the matrix for mounting and subsequent staining. None of these approaches is really practical for the study of cells in situ, nor do they offer a permanent record of the entire culture for later comparative studies. Furthermore, although described methods will allow a degree of morphological recognition, this is achieved at the cost of disrupting cellular relationships. We have recently developed a technique which makes it possible to realise these objectives.

In recent years there has been a steadily increasing application of the in vitro culture of marrow for studying the physiological and pathological features of haematopoiesis, and improvements in methodology regularly appear in the literature. We have analysed our experiences with this assay system and report these in the context of clinical applications of the method.

**MATERIAL AND METHODS**

The 414 studies which were carried out are analysed in Table I. Underlayers were prepared each week in 35-mm Petri dishes (Falcon 3001) containing 1 × 10⁶ normal leucocytes/ml of 0.5% agar (Difco). Marrow or blood cells to be studied were harvested from dextran sedimentation and overlaid as a monocellular suspension in 0.5% agar at a concentration of 2 × 10⁶ cells/ml. Petri dishes were incubated at 37°C in a humidified atmosphere containing a final concentration of 5% carbon dioxide. All studies were done in triplicate and included control cultures using NIH-conditioned medium derived from human embryonic kidney (J. M. Bull — personal communication). The plating efficiency was determined by counting the colonies and clusters on days 10, 12 and 14, using either an inverted microscope with phase-contrast illumination or preferably a stereoscopic dissecting microscope with an incident light source. Parallel cultures were set up in agar without underlayer or additional conditioned medium to

<table>
<thead>
<tr>
<th>TABLE I. ANALYSIS OF 414 STUDIES CARRIED OUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnoses</td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Preleukaemia</td>
</tr>
<tr>
<td>Acute non-lymphoblastic leukaemia</td>
</tr>
<tr>
<td>Acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>Acute non-lymphoblastic leukaemia in remission</td>
</tr>
<tr>
<td>Acute lymphoblastic leukaemia in remission</td>
</tr>
<tr>
<td>Myeloproliferative syndrome — bone marrow</td>
</tr>
<tr>
<td>Myeloproliferative syndrome — peripheral blood</td>
</tr>
<tr>
<td>Multiple myeloma — AB serum</td>
</tr>
<tr>
<td>Neutropenia</td>
</tr>
<tr>
<td>Aplastic anaemia</td>
</tr>
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</table>
monitor the spontaneous growth rate and the colony:cluster ratio. Marrow cells from patients with myeloma were washed in tissue culture medium and then cultured in either autologous or AB serum to demonstrate the presence of humoral inhibitors to in vitro growth.

At the completion of the culture period, the mean number of colonies and clusters for each plate was determined and the ratio recorded. The entire Petri dish was then flooded with fixative; the agar discs were freed and transferred to a watch glass with excess fixative. After separation from the underlayer, which was discarded, the overlayer was mounted on a glass slide, air-dried and stained using a variety of biological dyes and cytochemical reactions. Morphological screening was done using Romanowsky, haematoxylin-eosin and methyl green-pyronin stains. When indicated, esterase, Sudan black B or myeloperoxidase reactions were employed. For ultrastructural studies, colonies were selected from plates that had been fixed in 3% glutaraldehyde (pH 7.2); these were processed and sectioned at 400 Å on an ultramicrotome. Grids were viewed on a Siemens electron microscope and appropriate fields were photographed.

**RESULTS**

The results of the 414 studies at the 12-day period of culture are seen in Table I and Fig. 1. From normal marrow a mean of 29.9 colonies (SEM ± 2.49) and 39.1 clusters (SEM ± 3.59) was found; the colony:cluster ratio is 1 : 1.3. As the culture ages, the morphological features of the cells change from neutrophils (Fig. 2) to a period when macrophages are also present, until finally only macrophages are found (Fig. 3).

Samples obtained from bone marrow transplant donors gave results in the normal range. However, in two cases a marked decrease in plating efficiency was found and neither of the recipients achieved engraftment despite the presence of adequate numbers of nucleated cells, the latter being the usually accepted criteria for graft adequacy.

In patients with preleukaemia there is a marked reduction in overall plating efficiency reflected in the colony count of 2.75 (SEM ± 0.85) and clusters of 15 (SEM ± 4.24). The colony:cluster ratio may initially be within the normal range but typically a disproportionate number of clusters grow, resulting in the abnormal ratio of 1 : 5.5.

In patients with acute non-lymphoblastic leukaemia, both plating efficiency and colony formation are markedly reduced. Colony formation was 5.2 (SEM ± 1.34), but abundant cluster formation, 63.94 (SEM ± 12.54), was seen.

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**Fig. 2.** Granulocyte colony (× 1000). The cytoplasm is finely granular and stains a delicate pink using the methyl green-pyronin method. Seven-day culture from normal human marrow.

**Fig. 3.** Macrophage colony (× 1000). Note nuclear detail and granular cytoplasm due to phagocytosis of the agar. This granularity appears as a brilliant orange colour in methyl green-pyronin-stained preparations. Fourteen-day culture from normal human marrow.
resulting in profound disturbance in the colony: cluster ratio. A similar pattern was seen in acute lymphoblastic leukemia, where subnormal colony and cluster formation was found (8.17 ± 2.59 and 23.83 ± 3.6 respectively). For the two morphological variants the ratios were 1:12.3 and 1:3 respectively. These deviations from normal are statistically significant (P<0.05).

Patients with adult acute leukemia, whether non-lymphoblastic or lymphoblastic, who had achieved and maintained complete remission, are characterized by normal mean colony counts of 30.87 (SEM ± 2.85) and 22.43 (SEM ± 2.99) respectively. Similarly, cluster counts were 56.02 (SEM ± 3.86) and 47 (SEM ± 6.63) for the two leukemic variants. Colony:cluster ratios for the two groups of leukemic patients in remission were 1:1.8 and 1:2.1. None of the values deviates significantly from normal (0.04<P<0.05).

In one patient with acute non-lymphoblastic leukemia, a transient marked increase in overall growth with abundant colonies preceded the more usual changes in ratio by 3 months (Fig. 4) and the patient subsequently relapsed. This pattern may be another manifestation of occult disease and should alert physicians to the possibility of impending deterioration. The change in growth pattern was associated with an alteration in the character of the colonies, which were large and loosely packed with cells, a feature that is quite distinct from the usual density encountered during remission.

In patients with the myeloproliferative syndrome the pattern did not initially differ significantly from the normal; no growth was obtained from blood while marrow yielded mean counts of 42.55 colonies (SEM ± 10.85) (0.7<P<0.8) and 46.3 clusters (SEM ± 8.94) (0.6<P<0.7). However, with progression of the disease, a marked contrast from normal peripheral blood emerged. Normally unconcentrated blood grows virtually no colonies or clusters, whereas in the myeloproliferative syndrome 26.32 colonies (SEM ± 3.54) and 41.47 clusters (SEM ± 8.38) were found. The onset of accelerated phase or blastic transformation was characterized by a decrease in plating efficiency in the blood and a prominent disturbance in colony:cluster ratio, a finding that closely parallels that obtained from leukemic marrow.

In myeloma, colony formation is generally depressed to a level of 17.61 (SEM ± 2.12) while cluster counts are normal at 34.08 (SEM ± 4.19). It is noteworthy that no significant difference is demonstrable when washed marrow cells from patients with myeloma are cultured in either autologous or in AB serum, indicating that humoral inhibition of granulocyte:macrophage colony formation is unlikely to be found in the patient's serum.

In neutropenic subjects, colony:cluster ratio was not significantly different from normal, with values of 12.8 (SEM ± 2.87) and 35.83 (SEM ± 1.78) respectively. These observations indicate that peripherally acting mechanisms were present in our patients rather than production defects in the stem cells, where poor growth would have been anticipated.

In aplastic anemia, colony formation was markedly depressed to 2.63 (SEM ± 1.15) with cluster counts of 23.38 (SEM ± 5.71), findings characteristic of a stem cell lesion. It was not, however, possible to exclude the possibility that these findings may reflect inhibition occurring on the basis of cell-to-cell interaction taking place in the culture.

**DISCUSSION**

The demonstration that progenitor cells (CFU) of the granulocyte:macrophage lines derived from samples of bone marrow have the capacity to clone *in vitro* gave considerable impetus to the use of this technique for the study of both normal and abnormal haematopoesis. Before the availability of semi-solid agar culture methods, the complex nature of the regulatory mechanisms operating within the intact bone marrow and the difficulty of isolating segments of the differentiation sequence made analytical studies almost impossible. Although liquid cultures were used, they had many of the same limitations that applied to the *in vivo* experiments. For example, the effects of any manipulation were seen only at the end of a chain of related events, and it was difficult to segregate and manipulate individual phenomena occurring during haematopoietic proliferation and maturation. In contrast, the suspension of progenitor cells in a viscous matrix makes it possible to explore some of these physiological processes and their regulation at cellular and humoral level.

The current technique has been developed from the early studies of Puuzlik and Sachi and Bradley and Metcalf, with subsequent adaptation of the method to human material by Pike and Robinson. Further refinements have followed with the preparation of the colony-
stimulating factor (CSF) from a variety of different tissue sources, and standardization can be achieved by titrating varying quantities of this material against fixed numbers of CFU from bone marrow that has been previously characterized and then stored. The regular use of such dose-response curves and the inclusion of an international standard (J. M. Bull — personal communication) will provide quality control and enable the results between laboratories to be compared.

In physiological terms the culture of bone marrow in agar has been instrumental in demonstrating that a relationship exists between the granulocyte and the macrophage: monocyte system, and this, in turn, has led to the enunciation of a hypothesis for the homeostatic regulation of granulocyte mass.8,9 Basically it appears that macrophages elaborate a CSF that promotes granulopoiesis and as the granulocyte pool expands it produces increasing quantities of a second humoral factor or chalone, having colony-inhibiting activity (CIA) which, in an adequate concentration, will suppress granulopoiesis.8,10 Since this is a dynamic system, a critical point will be reached where the balance between these two factors again alters, with the result that macrophage-derived CSF becomes dominant and CFU favours neutrophil production. It has also been suggested8 that bacteria may play a pivotal role in granulocyte production by serving as a stimulus to increase in CSF elaboration, and as the newly formed cells remove the antigen, the CSF level falls and the neutrophil generation decreases. More recently it has been shown that a similar negative feedback loop may be mediated by macrophage-derived prostaglandin E production. From these studies it is evident that in vitro bone marrow culture has an important role to play in elucidating the mechanisms that interact to control granulopoiesis.

In bone marrow transplantation graft rejection occurs in a number of patients, and this is usually on the basis of prior exposure to blood products with sensitization and iso-antibody formation. Alternatively, it is possible that insufficient numbers of stem cells may be infused and, here also, engraftment will not be achieved. It is a simple matter to culture a sample of the donor graft and thus ensure that this does not happen. We have found at least one clear example and a second probable case where the patient received an adequate graft on the basis of numbers (3.2 x 10^6 nucleated cells/kg), but very poor growth was obtained in culture, and haematopoietic reconstitution did not occur. In vitro studies are thus a simple precaution to recognize those marrow donations where a paucity of stem cells may be expected to produce suboptimal results.

The study of pathologically disordered cell regulation is exemplified by the haematological malignancies. In the patients with preleukaemia there is a variable but usually marked reduction in plating efficiency accompanied by a disturbance in colony: cluster formation. The cytological detail of the individual cells in the aggregates and their ultrastructural examination remains an area for further development. Despite the apparent diagnostic value of culture studies in the preleukaemic patient, their place remains controversial, since clinical progression of the disease is still considered the essential criterion for initiating therapy. Nevertheless, if it can be shown that the combination of culture and morphology, perhaps combined with cytogenetics, will reliably predict the development of leukaemia, then the approach to treatment may be expected to change.

Similarly, bone marrow obtained from patients with acute leukaemia, both untreated and during relapse, grows with a characteristic pattern in agar. There is a marked reduction in overall growth or plating efficiency, colony formation is depressed and a variable number of clusters is evident. These findings are more prominent in lymphoblastic than in non-lymphoblastic leukaemia. It is notable that morphological relapse may only become evident many months after these abnormalities are manifest in culture, and the latter frequently appear at a time when the patients are clinically and haematologically normal. In this context we have encountered a leukaemic patient who, in complete remission, demonstrated a transient period of generalized acceleration in growth, evident many months before even colony: cluster ratio was disturbed, and without any pathological blasts being demonstrated in the blood or marrow; overt disease became obvious 6 months later. The explanation for this finding is uncertain; it might theoretically represent rapid expansion of the leukaemic clone or, alternatively, the response of normal progenitor cells to markedly enhanced humoral drive. The latter finding is favoured since the colony: cluster ratio was maintained, which contrasts with the more usual situation in leukaemia where the cells grow poorly or not at all, and tend to form clusters rather than colonies.

The best method of culturing leukaemic cells remains controversial, and despite reports that this is possible,11 there is no uniformity of agreement that the tumour cells will consistently grow in culture. Clearly, such an achievement would potentially provide a most sensitive measurement for the detection of residual leukaemia and be vital in defining early relapse. The scope of the method can be expanded by using methods to display the distinctive morphological features and the kinetic behaviour of leukaemic cells as compared with normal CFU. Detailed examination of cellular content within the clusters and the colonies as they exist in situ has been difficult, largely because no simple method has been available for this study, although recent experience with a different technique12 may help to resolve the problem. Similarly, the ability to store and maintain the viability of leukaemic bone marrow progenitor cells offers a practical opportunity to examine the influence of these cells in a number of different ways. For example, it should be possible to quantitate and characterize their production of CSF and to define its influence on normal CFU in culture. Conversely the response of these malignant cells to exogenous stimulating factors remains of interest. In addition, more sophisticated control mechanisms can be tested, such as interaction of the cellular level between normal and leukaemic cells using co-culture systems.

A third group of haematological neoplasms explored with this technique are those of the myeloproliferative syndrome. The pattern by which the marrow growth
changes as the disease evolves is now well defined and in chronic granulocytic leukaemia and myelofibrosis it is characterized by dislocation of the progenitor cell population from the marrow to the peripheral blood, a time sequence that has distinct diagnostic possibilities. Furthermore, a defect in plating efficiency and a change towards a leukaemic type of colony: cluster ratio develop as acceleration or blastic transformation occurs. Of equal interest is the potential of this technique to display the interrelationship between haematopoietic and stromal cells in vitro, thus providing a model with which to characterize myelofibrosis and perhaps to understand better the controversy about its pathogenesis.3 This argument has recently been given sharper focus by reports that stromal changes are secondary to the development of the haematopoietic lesion and are not part of the primary neoplasm.

The application of in vitro marrow culture to demonstrate humoral inhibitors is illustrated by examining marrow from patients with myeloma plated in either autologous or AB serum. Failure to depress colony formation excluded a humoral inhibitor as a major cause of the pancyclopenia that may be present in these patients at diagnosis. The concept that defective haematopoiesis may be on the basis of cell:cell interaction can be tested by co-culture experiments. The latter possibility has its basis in the possibility that sub-sets of T lymphocytes may possess suppressor activities which will be reflected in pancyclopenia or marrow hypoplasia.

Cloning techniques are helpful in formulating a functional or kinetic classification of the neutropenia. Thus it is possible to separate defective production clearly from situations where accelerated loss of mature cells occurs peripherally, as in classic immune neutropenia. In the latter situation, culture of the bone marrow shows normal or enhanced plating efficiency with retention of a normal colony: cluster ratio. This information is of relevance to the clinician who must direct investigation and therapy quite differently in the two groups of patients.

The application of in vitro culture systems to the study of patients with aplastic anaemia may help to unravel aetiological mechanisms in this heterogeneous group of diseases. In patients with a stem cell defect, poor plating efficiency is typical and is usually accompanied by a disturbance of colony: cluster ratio; here bone marrow transplantation remains the preferred therapy. In theory, any purely humoral cytotoxic factors could be identified by comparing cultures of progenitor cells in AB serum with those in autologous serum after initial washing to free the CFU of attached antibody. In the light of recent studies showing that immunologically mediated suppression of haematopoiesis may involve a subpopulation of patients with bone marrow aplasia requiring only immunosuppressive therapy, the ability to select these correctly by in vitro culture may come to assume still greater diagnostic importance. Clearly, then, the current objectives are to separate immunological lesions from those due to defective stem cells and to clarify the role of the micro-environment in the pathogenesis.

It is concluded that in vitro culture systems for the study of bone marrow have substantial clinical potential but require further development. Technically, there is the need to standardize methodology and refine morphological and infrastructural studies of the cells and combine these with more sophisticated techniques such as cyto genetics. Physiologically, these methods provide a practical approach to the isolation and study of cellular and humoral effects in haematopoiesis, including interactions between stromal and haematopoietic stem cells. In clinical practice they have a role to play in the characterization of bone marrow grafts and form part of the current diagnostic tests for a range of haematological malignancies, including pre-leukaemia, early leukaemic relapse or the presence of minimal residual disease. Similarly in patients thought to have the myeloproliferative syndrome, the demonstration of CFU in the peripheral blood is a valuable ancillary to the diagnosis and provides one means of monitoring possible leukaemic transformation. The further applications of this method are numerous and include characterization of patients with neutropenia and bone marrow aplasia.

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Bone Marrow Culture in vitro.
A Technique for Analysis and Permanent Recording of Cellular Composition

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The in vitro cloning of haematopoietic progenitor cells derived from blood or bone marrow is now an established technique for the study of normal and abnormal blood formation. In semi-solid agar the results are conventionally recorded as the number of clusters or colonies that grow on the plate under controlled culture conditions. However, the demonstration of detailed morphology within these cellular aggregates remains unsatisfactory. Aspiration techniques are cumbersome and invariably disturb cellular relationships within the supporting matrix while supravital staining is limited by variable uptake of dye by the agar.

We describe a method in which the entire cell-containing layer is removed from the Petri dish, fixed, and after mounting on a glass-slide, is air-dried. This preparation stains well with a wide variety of biological dyes, is minimally influenced by background colouration of the culture medium and excellent demonstration of morphologic detail is possible. A permanent record of the cellular composition of the culture is easily obtained by mounting the stained agar disc.

Key words: CFU colony morphology – colony composition

Cloning techniques for the in vitro study of bone marrow were initially attempted in mice (1, 2) and shortly thereafter applied to the study of haematopoiesis in humans (3). Subsequently, many of the technical details have been defined, including the characterization of the conditions necessary for culture of the different haematopoietic cell lines and the identification of a variety of effective colony stimulating factors (4). Nevertheless, the method is limited by lack of a simple method for the study of cellular morphology as it exists on the plate. Phase contrast or supravital staining can be used, but resolution of cytologic detail is poor and differential staining not practical. Alternatively, colonies or clusters may be aspirated with a pipette and following concentration on a slide, using a cytocentrifuge, are stained and examined (5, 6). These techniques are cumbersome and disturb the relationship of the cells within the supporting matrix.
Furthermore, most of the methods in use are affected by marked absorption of the stain by the wet supporting matrix.

We have developed a technique in which the complete agar disc containing the colonies and the clusters is removed from the Petri dish and fixed, mounted on an albumin-coated slide and, air-dried. This preparation is suitable for staining with a wide variety of biological dyes resulting in excellent demonstration of morphologic detail while maintaining intact cellular relationships within the supporting matrix. The method is simple and has the additional advantage in that mounting will provide a permanent record of cellular composition and growth pattern of the entire culture as it occurs in situ.

METHODS

Culture technique

The method is based upon that of Pike & Robinson (3) in which underlayers are prepared fresh each week from normal leucocytes. 1 x 10⁷ white cells are suspended in 9 ml McCoy's tissue culture medium containing 20% foetal calf serum, 100 µg penicillin, 100 µg streptomycin and 1 ml of 5% agar (Difco). 1 ml of this mixture is pipetted into a 35 mm Petri dish (Falcon 3001) providing 1 x 10⁷ normal leucocytes in 0.5% agar.

1 ml of 0.3% agar containing 2 x 10⁷ cells is pipetted onto the feeder layer and the plate incubated at 37°C in a fully humidified atmosphere containing 5% carbon dioxide in air. Cultures are set up in triplicate and read at 7, 10, 12 and 14 days using either an inverted or a stereoscopic dissecting microscope. Plating efficiency is expressed as the average number of colonies or clusters on the three plates at each of the time intervals and recorded as a ratio.

Fixation

The entire plate is flooded with fixative and underlayer and overlayer separated from the edge of the Petri dish by simple rotation. In the event that the two layers adhere peripherally, a 32 mm disc is cut free, using a cork borer of suitable diameter. The underlayer and overlayer are transferred with excess fixative to a 10 cm watch glass where they are easily separated and the underlayer discarded. The overlayer is allowed to fix for 1 hour at 22°C; the fixative varies and is determined by the particular staining technique that will subsequently be employed. Three percent glutaraldehyde in 0.1 M cacodylate-hydrochloric acid buffer at pH 7.2 is used for Romanowsky, haematoxylin-eosin and methyl green-pyronin staining. After removal from the glutaraldehyde the agar disc is rapidly dehydrated and air-dried. Zenker's fluid gives equally good fixation but its use is time-consuming in that removal of mercuric chloride and subsequent bleaching introduce additional steps and add little to the quality of the glutaraldehyde fixed preparations. Analytical grade methanol provides a practical alternative when methyl green-pyronin, periodic-acid Schiff or myeloperoxidase staining are used. Where chloroacetate or non-specific esterases are to be demonstrated, buffered formalin to acetone is preferable and for Sudan black B a 10% formalin ethanol gives optimal results. After fixation the agar disc is mounted on an albumin-coated glass slide and air dried at 22°C overnight.

Stains

Romanowsky staining is satisfactory when the dried slides are immersed for 6 hours in phosphate buffer at pH 6.8 containing a final concentration of 1% stain. Giemsa, Wright or May-Grünwald may be used independently but we have found that an equal mixture of the three gives better results. After 4–6 hours the slides are removed from the dilute stain, rinsed in buffer and rapidly dried on a blood spinning centrifuge, although a small hand operated hair dryer is adequate. Granule differentiation can be modified by increasing the staining time up to 18 hours and then differentiating in ethanol containing 0.5% rosen and 0.05% acetic acid (Coleman, personal communication 1975) before air drying as above. To retain tinctorial characteristics passage through alcohol for dehydration should be avoided. The dry preparation is rinsed in xylol and mounted under DPX (BDH Chemicals, Poole, UK).

Haematoxylin-eosin, methyl green-pyronin, periodic-acid Schiff and toluidin blue stains are carried out according to standard histochemical procedures (7).

Cytotoxic and histochemical studies include Sudan black B (7), acid and alkaline phosphatase (7), chloroacetate and non-specific esterase (8, 9) and myeloperoxidase (10).
Electron microscopy

Material for ultrastructural study is selected under direct vision following gluteraldehyde fixation and the 2 mm discs containing the colonies are cut free from the matrix using a core borer, removed from the plate, rinsed in 0.1 M phosphate buffer, pH 7.2, overnight and post-fixed in 1% osmium tetroxide in veronal buffer at pH 7.2 for 15 minutes. After washing in 0.1 M phosphate buffer for 1 hour, the discs are pre-stained with 2% uranyl acetate in 10% acetone, dehydrated and embedded in Spurr’s resin (TAAB laboratories, Reading, UK) at 70°C overnight. The sections are cut on an ultra-microtome at 400–500 Å, stained with saturated solution of uranyl acetate, rinsed, post-stained with 0.2% lead acetate and viewed on a Siemens microscope; suitable fields are then selected and photographed.

RESULTS AND COMMENTS

The preservation of the entire plate is easy to achieve and a linear relationship demonstrated to exist between the number of colonies and clusters present in unfixed culture and those subsequently seen on the stained preparations.

The fixatives were of importance and 3% gluteraldehyde was found to be the most practical*. Zenker’s solution also gives good preservation of cellular detail but is cumbersome and was not found to be superior to gluteraldehyde. Methanol generally produced high quality plates but was less predictable in its penetration and fixing characteristics so that cell margins were often indistinct, and granules less well preserved than with either gluteraldehyde or the more tedious Zenker fixation. Methanol was, however, particularly effective with methyl green-pyronin staining. The use of special fixatives for the cytochemical stains does not differ substantially from standard methods employed in the study of blood or bone marrow.

The Romanowsky stains were consistently the most useful since they allow direct correlation with the original samples. Combined with gluteraldehyde fixation the cells were easily recognised in that cytoplasmic and nuclear outlines were distinct and granules were prominent. Variations in the concentration of dye, the staining period and the use of differentiation allowed accentuation of individual characteristics. However, for routine use, a 1% final concentration of mixed Romanowsky dyes and an exposure time between 4 and 6 hours, without differentiation, was adequate. It is important to avoid dehydration in the alcohols since this results in less satisfactory preparations than rapid air drying before mounting.

Haematoxylin and eosin staining was useful in demonstrating nuclear detail, but cytoplasmic structures were less clearly defined. Thus, granule demonstration, although feasible, compared unfavourably with the Romanowsky stains.

Methyl green-pyronin provided a good general stain in that it combined excellent demonstration of morphologic features and distinct differences in staining. Macrophages (Figure 1) stained brilliant orange with prominent cytoplasmic material while the granulocytes (Figure 2) stained a delicate shade of pink although individual granules were poorly appreciated. The macrophages characteristically contained ingested agar and this substance probably accounts for the distinctive colour produced with pyronin.

On the basis of our experience this simple battery of three stains appears suitable for most of the screening, but in certain circumstances additional cytochemical studies are helpful. To ensure uniformity of sampling the plate is divided into quadrants so that comparable portions can be examined with the different techniques.

Myeloperoxidase, chloroacetate ester-
ase and non-specific esterase are a useful combination facilitating differentiation between granulocytes and cells of the monocyte with macrophage line. In addition sudan black B is easily applicable to the fixed material and will broaden the scope for morphologic study. Periodic-acid Schiff provides acceptable staining characteristics but is of limited value apart from demonstrating basic morphologic features in another way. Similarly, toluidin blue can be employed to show granules or phagocytosed agar in macrophages.

Of note is the fact that most of the staining methods used with this technique are not markedly influenced by background staining and this contrasts with experience where unfixed or wet matrix is present. Any staining that does occur can be diminished in intensity by relatively minor modifications in dye concentration. Under these circumstances colour photographic recording rather than preservation of this disc is an alternative means of securing a permanent record of the culture.

In certain circumstances more detailed examination of the cells may be required and here electron microscopy will provide an excellent preparation (Figure 3) to complement the morphologic and cytochemical studies. For example, attempts to clone mouse lymphocytes yielded cells which could not be confidently distinguished from monocytes; ultrastructural studies resolved this dilemma.

The technique is also well suited to more sophisticated examination of the cells including demonstration of specific immunoglobulin production by means of peroxidase: anti-peroxidase technique and kinetic studies using autoradiography.
result in excellent demonstration of morphologic detail within the cellular aggregates as they exist in situ. A further advantage is that the stained plate provides a permanent record of the culture and is suitable for exchange of information between centres.

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Clonogenic growth patterns correlate with chemotherapy response in acute myeloid leukaemia

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Abstract
Cytosine arabinoside and anthracycline-containing regimens induce remission in upwards of 60% of previously untreated patients with adult acute myeloid leukaemia (AML). Despite this, in addition to primary drug resistance, the majority of these patients relapse. Reliable methods for uniformly recognising these two subgroups at presentation do not exist and therefore a further attempt has been made to relate in vivo toxicity, using a clonogenic assay, to clinical outcome. In 10 normal controls and 12 chemotherapy naïve cases, mononuclear cells harvested by density gradient separation were re-suspended at a concentration of 2 x 10⁶/ml and quadruplicates of 250 μl per well cultured in methylcellulose containing foetal calf serum and phytohaemagglutinin stimulated leucocyte conditioned medium. Cell kill was determined for cytosine arabinoside, daunorubicin and etoposide either singly or in combination using both a pulsed and continuous exposure. Aggregates were scored after seven days and three distinct patterns recognised. The patients all received the same drugs in a standard protocol and achievement of complete remission correlated with growth pattern. The survival of normal marrow colony-forming cells or GM-CFUc and the leukemic equivalent designated L-CFUc were assessed and a sensitivity index (SI) determined as a ratio of these two values in which more reproducible results were found when the drug was continuously present. It is concluded that the microculture technique is feasible and clearly demonstrates chemotherapy effect but no correlation was demonstrated with clinical outcome. This is a negative pilot study and, as a means of recognising drug sensitivity or resistance, should be discarded in favour of currently available molecular techniques.

Keywords: Cell cycle, cytosine, acute myeloid leukaemia, sensitivity index

Introduction
A significant number of patients with acute myeloid leukaemia (AML) will achieve complete remission with currently employed treatment regimens typically containing cytosine arabinoside with maximum effect on cell cycle during nucleic acid synthesis and anthracycline antibiotic intercalating adjacent base pairs and inhibiting DNA and RNA with subgroups of patients having further benefit from the epipodophyllotoxin inhibiting topoisomerase II in a cell cycle dependent manner with specificity in late S and early G II phases [1–3]. Despite careful selection of cases, using a variety of prognostic factors [4], a number will have primary drug resistance [5] and the majority will randomly relapse in the post chemotherapy period often having achieved morphologic complete remission [6]. While no standard clinical or laboratory features reliably predict this event [7] increasing attention is being given to immunophenotyping [8], gene rearrangement with much of
the early work focussed on the lymphoblastic tumours [9,10] as well as minimal residual disease [11,12].

Past attempts have also examined in vitro assays for clonogenic cells particularly those reflecting the leukaemic lineage and designated CFUc-L [13,14]. This approach has the potential to define proliferate capacity, growth requirements and drug sensitivity of occult disease [15,16]. To further explore this application, the previous observation of poor leukaemic growth in agar has been compared to methylcellulose as the matrix [17,18]. Secondly, the agents were tested singly and in combination at different incubation times [19,20]. Drug sensitivity was defined by results after continuous exposure in comparison to sensitivities determined at 1h pulse and both correlated with initial clinical response in individuals with acute myeloid leukaemia.

Materials and methods

Patients

Twelve untreated patients with newly diagnosed AMLs, classified according to the French–American–British criteria were contrasted to 10 hematologically normal controls [21,22].

Treatment

Patients received a 7-day continuous infusion of cytosine arabinoside (100 mg/m²), 7 days of etoposide as a half-hour bolus (100 mg/m²) and daunorubicin on the first 3 days (45 mg/m²). Complete remission was defined as the disappearance of blasts from the peripheral blood and normal haematopoesis in the bone marrow. Non-responders were individuals who had residual disease after two courses of induction therapy.

In vitro culture assay

Bone marrow was aspirated from the posterior iliac crest into preservative-free heparin and mononuclear cells (MNC) recovered following density gradient separation by Ficoll-Hypaque (Sigma catalogue No 1077-1, density 1.077 g/ml), washed twice with Iscove’s Modified Dulbecco’s medium (IMDM- Gibco, UK) and re-suspended with IMDM with 30% foetal calf serum (FCS) (Delta Bioproducts, SA). Based on previously described methods [23,24] 2 × 10⁵ MNC/ml, contained in 2 ml of IMDM and having a final concentration of 0.9% methylcellulose, (Sigma, UK), 20% FCS and 10% phytobhaemagglutinin-leucocyte condition medium [25] (PHA-LCM) (Welcome, UK) were plated in 250μl per well in quadruplicate (NUCC Multidish Catalogue No. 176740, Denmark) and the mean value of the four micro wells reported. The cultures were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide for 7 days and then scored using an inverted microscope. Aggregates of greater than 40 cells were defined as colonies and between 10 and 40 as clusters.

In an attempt to further enhance sensitivity growth patterns were reassessed by dividing them into type A containing <20 colonies and <200 clusters, type B with <20 colonies and between 200 and 400 clusters and type C with >20 colonies and >200 clusters identified subgroups requiring additional courses of induction chemotherapy.

Drug preparation and cytotoxicity testing

These agents were freshly prepared immediately before culture and had been shown to be stable at −70°C for 3 weeks [26,27]. Final concentration with cytosine arabinoside [28] (Upjohn, UK) was 12.5 μmol/l and etoposide (Bristol Myers/Squibb, SA) 20 μg/ml. These concentrations approximate those previously established as being optimal for in vitro chemotherapy testing [29].

In pulse experiments, 6 × 10⁵ MNC/ml were pre-incubated in liquid suspension culture with the appropriate concentrations of each respective agent or drug-free control media. After 1h the cells were washed twice with IMDM, re-suspended with 20% FCS in IMDM. After which cells counts performed and viabilities determined by trypan blue exclusion. 2 × 10⁵/ml of cells were then plated at 250 μl in methylcellulose as the immobilising agent.

In continuous exposures, 2 × 10⁵/ml of cells were plated directly into the methylcellulose containing the drug and incubated for 7 days. Control cultures containing patients’ cells without drugs were set up simultaneously. Patterns derived from normal marrows were used to establish survival in the GM-CFUc assay from the leukemic cells and so obtain a sensitivity index (SI) [30].

Calculations

Using an inverted microscope, the cultures were scored on day 7, defining the number of surviving colonies, percentage inhibition from the leukaemic aspirates compared to controls without the drugs [31] and the SI determined when the two groups were compared based on the mean value of the 10 normal marrows.

\[
\% \text{ survival} = \frac{\text{cell survival after drug exposure}}{\text{cell survival with no drug exposure}}
\]

The sensitivity index of group 1 or leukemic patients compared to group 2 or normal controls for
Table I. Patient and disease profiles with treatment outcome for 12 cases of acute myeloid leukaemia.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>FAB</th>
<th>CR</th>
<th>Treatment</th>
<th>Other comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>M</td>
<td>M3</td>
<td>No</td>
<td>CTRIV</td>
<td>Died</td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>F</td>
<td>M1</td>
<td>No</td>
<td>CTRIV</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>F</td>
<td>M4a</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 2, Consolidation × 2. Died^2</td>
</tr>
<tr>
<td>4</td>
<td>35</td>
<td>M</td>
<td>M5a</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 2, Consolidation × 2, BMTX</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>F</td>
<td>M1</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 1, Consolidation × 2</td>
</tr>
<tr>
<td>6</td>
<td>17</td>
<td>M</td>
<td>M1</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 1, Consolidation × 2. Died^d</td>
</tr>
<tr>
<td>7</td>
<td>47</td>
<td>M</td>
<td>M3</td>
<td>No</td>
<td>CTRIV</td>
<td>Died^d</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>M</td>
<td>M2</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 2, Consolidation × 2</td>
</tr>
<tr>
<td>9</td>
<td>58</td>
<td>F</td>
<td>M1</td>
<td>No</td>
<td>CTRIV</td>
<td>Induction × 1, Died</td>
</tr>
<tr>
<td>10</td>
<td>34</td>
<td>F</td>
<td>M2</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 1, Consolidation × 2</td>
</tr>
<tr>
<td>11</td>
<td>49</td>
<td>M</td>
<td>M4</td>
<td>Yes</td>
<td>CTRIV</td>
<td>Induction × 1, Consolidation × 2</td>
</tr>
<tr>
<td>12</td>
<td>14</td>
<td>F</td>
<td>M1</td>
<td>No</td>
<td>CTRIV</td>
<td>Induction × 3, Died^d</td>
</tr>
</tbody>
</table>

CR: Complete remission, is defined as disappearance of all morphological evidence of disease in peripheral blood and <5% blasts in bone marrow; BMTX: Bone marrow transplant; FAB: Morphological classification according to the French–American–British co-operative group.

* Patient died during induction therapy. ^ Patient relapsed.

each drug was determined:

\[
SI = \frac{\% \text{ cells killed from group 1 (average of controls)}}{\% \text{ cells killed from group 2 (individual cases)}}
\]

In preliminary studies, the percentage survival of cells was low and the percentage of cells killed was found to be a more reproducible endpoint. Thus, a sensitivity index of 1.0 is defined as no difference in drug sensitivity between GM:CFUc and CFUcL. If the CFUcL shows greater killing or sensitivity to the drug than GM:CFUc, the SI was less than 1 and the converse applied when the SI was greater than 1.

Results

Of the 12 cases enrolled (Table I) in vitro studies of marrow cells and types of growth formed by their cells in culture (Table II) were correlated with subsequent response to therapy.

Of these 3 with the type A proliferation pattern achieved complete remission and 2 are alive. Type B was found in 1 a single individual who entered remission and another 2 who died. The former patient required 2 induction courses to reach the status, but subsequently relapsed and demised. Type C was found in 6 of whom 3 entered remission, and in whom two needed a second course of chemotherapy. There were two failures to respond to treatment regimen and one died during this process.

In summary, of the 12 individuals having acute myeloid leukaemia, all with type A growth achieved complete remission. Of those with type B and C aggregates in the clonogenic assay 4 achieved complete remission but were more resistant to the therapy regimen and all needed two courses to clear recognisable blasts.

Discussion

The in vitro growth patterns of leukaemic cells in agar or methylcellulose appears to yield prognostic information that is clinically useful and this can be

Table II. In vitro growth patterns.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Colonies number</th>
<th>Clusters number</th>
<th>Pattern</th>
<th>Complete remission</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>214</td>
<td>B</td>
<td>No</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>564</td>
<td>C</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>355</td>
<td>B</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>136</td>
<td>825</td>
<td>C</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>132</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>124</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>7</td>
<td>63</td>
<td>759</td>
<td>C</td>
<td>No</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>558</td>
<td>C</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>212</td>
<td>B</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>115</td>
<td>426</td>
<td>C</td>
<td>Yes</td>
</tr>
<tr>
<td>11</td>
<td>19</td>
<td>136</td>
<td>A</td>
<td>Yes</td>
</tr>
<tr>
<td>12</td>
<td>82</td>
<td>234</td>
<td>C</td>
<td>No</td>
</tr>
<tr>
<td>Normal range</td>
<td>2-78</td>
<td>275-581</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Colonies contain >40 cells per aggregate.
Clusters 10–40.
Figure 1. *In vitro* survival of cloned cells: 1 h pulsed exposure to drugs.

Figure 2. *In vitro* survival of cloned cells continuous exposure of drugs for 7 days.
obtained prior to the treatment in patients presenting with acute myeloid variant when comparison is made to GM-CFUc from normal donors. Thus the number of clones produced under these circumstances by the neoplastic clone (CFUc) is more variable, and there is a preponderance of small-sized colonies defined as clusters of fewer than 40 cells. These growth characteristics appear to be independent of the FAB sub-type, the patient’s age or sex and cytogenetic status of the marrow cells [32,33].

In the groups A and B, 4/6 patients did initially responded to therapy and achieved remission. Two died, one during induction, and one which was acute progranulocytic sub-type or M3 during induction and a second one after completing this phase of the chemotherapy. In group C, 3/6 also achieved remission but two needed a second induction course. Of the 3 patients that failed 2 did so during the initial cytotoxic drug infusion.

In this feasibility study, in vitro growth of marrow from patients with acute myeloid leukaemia has been the sole criterion for attempts to recognise distinctive patterns that may have predictive value for chemotherapy regimens. In groups A and B where proliferation in the clonogenic assay was poor prognosis appeared better. This is in contrast to studies by Curtis and colleagues [34–36], who were unable to correlate their findings with success or failure of remission induction. Conversely McCulloch and associates [37], and Brown and Carbone [38] have demonstrated that their culture method, which is similar to ours, does provide a predictive value in this context.

In the cytotoxicity assay normal human marrow colony-forming cells have nearly the same sensitivity as their leukaemic counterparts for each of the agents used individually or in combination [39]. The exposure for 1h may be too brief to reveal the sensitivity of cells to the drug regimen (Figure 1). In fact a prolonged contact time may be critical for all cell cycle-dependent drugs exemplified by a Cytosine arabinoside and etoposide (Figure 2). Thus it was found that when tested either individually or in combination the pattern for continuous exposure had similar cytotoxic effects on all the patients, and this was particularly true when used together. The figures further illustrate that response was significant in ex vivo but, in the same individuals, there was substantial difference in clinical outcome.

Clonogenic assays, as an approach to testing for drug sensitivity, have a number of limitations [39–41]. Taking these in consideration, coupled with the outcome in this feasibility study, it is concluded that they failed to reliably predict response in vivo. This may, in part, be a limitation of the small number of correlations presently reported. Also, dose response curves were not used but rather concentrations employed that had been established from prior publications. Taking all the available information together and in the context of other experience this assay cannot be recommended as a reliable predictor for correlating laboratory results with response in clinical setting.

Acknowledgements
Dr Ingrid Aronson and Ms Margie Veen are thanked for help with the laboratory studies. Appreciation is expressed to Professor Bob Lowenberg for advice in manuscript revision. Mrs Christine Dölling did the bibliographic research and the typing was expertly carried out by Mrs Henriette Meiring. Supported by the Haematological Research Trust and the Chairman’s Fund of the Anglo American Corporation.

References


The Secretion of Plasminogen Activators by Human Myeloid Leukemic Cells In Vitro

By E. Lynnette Wilson, Peter Jacobs, and Eugene B. Dowdle

Peripheral blood cell preparation from 23 normal subjects and 72 patients with acute and 32 patients with chronic myeloid leukemia were cultured in vitro and released plasminogen activators were analyzed. The quantity of plasminogen activator secreted by leukemic cells varied widely and could not be correlated with the clinical severity of the disease. Immunochemical and electrophoretic techniques have been used to show that normal peripheral blood granulocytes released exclusively urokinase-like plasminogen activator, whereas leukemic cells secreted either urokinase or a tissue activator-like enzyme. The molecular species of enzyme released by acute myeloid leukemic cells may serve as a diagnostic marker of relevance to the management of this disease, since patients with acute myeloid leukemia whose cells released only tissue plasminogen activator did not respond to combination chemotherapy. Tissue plasminogen activators released by leukemic cells may display an unusual electrophoretic pattern that resembles that shown by urokinase. Immunochemical procedures are therefore essential for the correct identification of these enzymes.

ALTHOUGH the chemotherapy of leukemia is based on sound pharmacologic principles, it remains empirical to the extent that individual patients may respond very differently to a particular chemotherapeutic regimen in terms of induction of remission, maintenance of remission, or incidence of undesirable side effects. It would therefore, be of value if additional laboratory criteria were available that could be used in conjunction with clinical and conventional histologic procedures to predict response to therapy.

It has recently been noted that plasminogen activator synthesis and release are inducible cellular functions that are subject to modulation by hormones, drugs, and other agents, many of which affect expression of the transformed phenotype. For this general reason, it seemed to us possible that the study of plasminogen activator synthesis in vitro would prove of value for the further characterization of human leukemic cells. Two recent observations have provided a more particular justification for this investigation. First, it has been demonstrated that normal granulocytes synthesize plasminogen activator; and second, it has been shown that human cells release plasminogen activators of two distinct immunological types—one similar to urokinase and the other similar to tissue activator. It was therefore of interest to determine (A) whether leukemic myeloid cells released both types of enzymes and, if so, (B) whether the molecular species of plasminogen activators released by myeloid leukemic cells differed in a way that would be nosologically useful.

In this article we present results of a series of studies showing that peripheral blood cells from patients with myeloid leukemia released plasminogen activators when cultured in vitro. Differences in molecular species of enzyme released by leukemic cells were observed. These differences appeared to have prognostic significance.

MATERIALS AND METHODS

Subjects

Normal blood samples were obtained from 23 healthy persons working in the laboratory. Leukemic blood samples were obtained from 72 patients with acute myeloblastic leukemia (AML) and 32 patients with chronic myeloid leukemia (CML) who attended the hematology service at Groote Schuur Hospital.

Diagnoses were based on histologic and histochemical examination of peripheral blood and bone marrow specimens. Romanowsky-stained preparations, cytochemical stains, and ultrastructural features were used to classify each specimen according to the French-American-British recommendations.

Fifty-eight patients with acute myeloblastic leukemia were treated with combination chemotherapy that included the epipodophyllotoxin, VP-16-213, cytosine arabinoside, and Adriamycin. Response to therapy was assessed by aspiration and trephine-biopsy of the bone marrow on the tenth day following completion of the cytotoxic chemotherapy. Induction of remission was judged to have been successful if marrow hypocellularity was achieved with subsequent regrowth of blast-free marrow that remained apparently normal for at least 4 wk.

Of the 58 patients treated in this way, 27 went into complete remission, 15 failed to enter remission, and 16 died before response to therapy could be assessed.

The 14 remaining patients that are identified in Table 1 as having had palliative or alternate therapy are individuals who, by virtue of their advanced age or associated clinical conditions, were judged to be unsuitable for combination chemotherapy.
LEUKEMIC CELL PLASMINOGEN ACTIVATORS

Table 1. Correlation Between Clinical Outcome and Molecular Species of Plasminogen Activator Released by Cultured Cells of 72 Patients With AML.

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Group</th>
<th>Response</th>
<th>Nature of Plasminogen Activator</th>
<th>TA*</th>
<th>UK</th>
<th>TA and UK</th>
<th>Unknown</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination chemotherapy</td>
<td>A</td>
<td>Assessment completed</td>
<td>TA*</td>
<td>0</td>
<td>22</td>
<td>3</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complete remission</td>
<td>UK</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No remission</td>
<td>(Subtotal)</td>
<td>(8)</td>
<td>(28)</td>
<td>(3)</td>
<td>(3)</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Died before assessment</td>
<td></td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Palliative/ alternate therapy</td>
<td>C</td>
<td></td>
<td></td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>45</td>
<td>5</td>
<td>7</td>
<td>72</td>
</tr>
</tbody>
</table>

*TA, tissue activator.
UK, urokinase.

Cells

Blood was taken by sterile venipuncture into tubes containing preservative-free heparin (Thromboliquine, Organon Teknika, Holland) to give a final concentration of 5 U/ml. Cells were fractionated by centrifugation with RPMI 1640 medium and centrifugation on a layer of Ficoll-Hypaque. Leukemic blast cells were harvested from the plasma-Ficoll interface and contaminating red blood cells removed by incubation for 5 min in 0.83% ammonium chloride (pH 7.4).

Cells were washed once by centrifugation and resuspension in RPMI and were then resuspended in RPMI containing 3% fetal calf serum (FCS) to give 4 \times 10^6 cells/ml. One milliliter aliquots of this suspension were incubated in 35-mm Falcon plastic tissue culture Petri dishes at 37°C in a humid atmosphere of 5% CO₂ in air. After 24 hr of incubation, the cells were suspended in fresh medium and the incubation was continued for a further 24 hr. At the end of this second period, the medium (harvest fluid) was collected by centrifugation and analyzed quantitatively and qualitatively for plasminogen-dependent proteases as described below.

In some cases, a caseinolytic plaque assay was used to obtain a rough estimate of the number of individual cells that synthesized plasminogen activators. In this procedure, the cells were washed and mixed with appropriate prewarmed solution to provide a final suspension of 6 \times 10^5 cells/ml in RPMI containing 0.8% agar, 1.3% solution of commercial instant non-fat dry milk powder, and 160 µg/ml purified human plasminogen. This suspension was run into prewarmed moulds formed from glass microscope slides separated by short lengths of thin wire. The preparations were then allowed to set at room temperature, when the slides were carefully separated and the gels incubated at 37°C in a humid atmosphere for plaques of lysis to develop (Fig. 1).

Plasminogen Activator Assay

Plasminogen activator in harvest fluid was assayed by measuring the plasminogen-dependent release of soluble radioactive fibrin degradation peptides from insoluble 125I-labeled fibrin-coated Linbro multwell plates. This was done exactly as previously described, save for the addition of 4 µg of plasminogen to each well instead of 2 µg.

Rabbit Antibodies to Human Urokinase and Tissue Plasminogen Activator

Rabbits were immunized by subcutaneous injection of commercially purified human urokinase or human tissue plasminogen activator secreted into the medium of a human melanoma cell line. The IgG antibody fractions from immune sera were prepared as pre-

Fig. 1. Plasminogen-dependent caseinolysis by leukemic cells. Acute myeloid leukemic cells were suspended at a concentration of 6 \times 10^5 cells/ml in agar gels containing casein. Gel A was supplemented with 160 µg/ml of plasminogen; plasminogen was omitted from gel B. Plaques of caseinolysis seen in gel A were photographed after 8 hr of incubation at 37°C.
Electrophoretic and Immunochemical Analysis of Plasminogen Activators

Molecular species of plasminogen activators present in harvest fluids collected from normal and leukemic cells were analyzed by 3 procedures previously described in detail.19

1. Harvest fluid samples were electrophoresed in 11% polyacrylamide gel slabs containing 0.1% SDS. The gels were then washed in 2.5% Triton X-100 to remove the SDS, and the bands of plasminogen activator activity were detected by plasminogen-fibrin-agar zymography in which plasminogen-dependent fibrinolysis was evident as clear lysis zones in the opaque fibrin indicator slab (Fig. 4).

2. Harvest fluid samples were assayed for residual plasminogen activator activity after treatment with serial dilutions of rabbit antibody to urokinase or to tissue plasminogen activator (Fig. 5).

3. Plasminogen activator in any given harvest fluid could be analyzed by a combined electrophoretic and immunochemical procedure in which specific antibody was added to a trough cut in the plasminogen-fibrin-agar indicator layer. The polyacrylamide gel slab containing electrophoresed plasminogen activators was then carefully layered over the indicator layer in such a fashion that adjacent electrophoretic tracks lay parallel to and on either side of the antibody-containing trough. Specific inhibition of individual activator bands could be seen in the antibody-rich agar adjacent to the trough (Figs. 6 and 7).

RESULTS

Fibrinolytic Activity Released by Cells Cultured In Vitro

When caseinolytic or fibrinolytic activity was released by normal or leukemic cells, this was invariably and completely plasminogen-dependent. This was evident as plaques of proteolysis in the casein-agar system (Fig. 1A) that were not seen when plasminogen was omitted from the indicator gel (Fig. 1B). Similarly, release of 125I-fibrin degradation peptides in the solid-phase radioenzymatic assay system was dependent on the presence of plasminogen.

Fig. 2. Inhibition of urokinase and tissue plasminogen activator by specific rabbit IgG antibodies. Samples of urokinase and tissue activator secreted by human melanoma cells were incubated with antibodies to urokinase (–—–), tissue activator (–—–), and ovalbumin (–—–) and assayed for residual enzyme activity in the 125I-fibrin plate assay. Undiluted antibody solutions contained 5 mg/ml of purified IgG.

Fig. 3. Release of plasminogen activator by cultured myeloid cells isolated from peripheral blood samples of patients with acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). Each point represents the mean result obtained from three cultures prepared from a peripheral blood sample obtained from a different patient. Immunochemical and electrophoretic techniques were used to identify the enzyme as urokinase-like (●), tissue-activator-like (□), or mixed (●).
Normal mononuclear cell preparations that remained on top of the Ficoll-Hypaque layer (i.e., lymphocytes and monocytes) released too little plasminogen activator for accurate quantitation or characterization.

Normal neutrophils that centrifuged through the Ficoll-Hypaque layer released easily measurable amounts of plasminogen-dependent fibrinolytic activity for purposes of molecular identification. Unfortunately, however, these cells survived poorly in culture.

The lack of a stable viable cell population over the duration of the experimental period made it impossible to express the quantity of enzyme released on the basis of cell number for purposes of comparison with leukemic cells.

Leukemic cells fared well in culture and, by the end of the 48 hr, more than 85% were still viable. These cultures therefore provided data that could be used both to determine the amount of enzyme released and the molecular species of enzyme that was synthesized.
four leukemic cell culture fluids. These were compared with urokinase (A) and tissue activator (F). The enzymes present in leukemic cell harvest fluids, B, D, and E, were electrophoretically similar to urokinase. Leukemic cell harvest fluid, C, contained plasminogen activators of three electrophoretically distinct types—one similar to tissue activator, one similar to urokinase, and a third gave a band of fibrinolysis corresponding to a mol wt of 100,000.

Antibody titrations showed that enzymes secreted by leukemic cells could be immunochemically identified as urokinase or tissue activator (Fig. 5). In this experiment, cells from one leukemic subject secreted urokinase-type activator (Fig. 5C), while cells from a second leukemic subject released enzyme that was exclusively tissue activator in type (Fig. 5D).

The immunochemical identity of the enzymes in cell harvest fluids could be more definitely identified using the combined immunochemical and electrophoretic procedures illustrated in Fig. 6. As shown in this example, one sample of leukemic cell harvest fluid contained activators that were completely inhibited by

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**Release of Plasminogen Activators by Leukemic Cells**

Cell preparations from 61 patients with AML and 32 patients with CML were cultured in vitro, and harvest fluids were taken for measurement of plasminogen activators released during the second 24 hr period. The results are presented in Fig. 3, from which it can be seen that the quantity of enzyme secreted varied widely over a 10,000-fold range from 0.001 U/10^7 cells/24 hr to 22.4 U/10^7 cells/24 hr. In 9 cases (7 AML and 2 CML), leukemic cells secreted too little enzyme to be detected in the fibrin plate assay.

**Molecular Species of Plasminogen Activators Released by Normal and Leukemic Cells**

Representative results of an electrophoretic analysis of the plasminogen activators released by cultured cells are given in Fig. 4, which shows a fibrin-agar indicator gel that was used to identify the enzymes present in

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**Fig. 6. Differential inhibition of plasminogen activators secreted by cells from patients with acute myeloid leukemia by antiurokinase antibody added to a trough (Ab) cut in a fibrin agar indicator gel. Tracks 1 and 2 contain plasminogen activator secreted by one leukemic individual, and tracks 3 and 4 contain enzyme secreted by a second leukemic individual. For experimental details see text.**

**Fig. 7. Inhibition of a 60,000 mol wt plasminogen activator secreted by cells from a patient with acute myeloid leukemia by anti-tissue-activator antibody added to a trough (Ab) cut in a fibrin agar indicator gel. Tracks 1 and 2 contain plasminogen activator secreted by cells from an individual with acute myeloid leukemia, and tracks 3 and 4 contain tissue plasminogen activator.**
antibody to urokinase (tracks A and B), whereas the harvest fluid from a second leukemic cell preparation (tracks C and D) contained one activator species of mol wt 60,000 that was inhibited by the antibody to urokinase, whereas the activators with mol wt of 70,000 and 100,000 were unaffected by the antibody to urokinase.

These procedures were used to show that cells from 15/72 patients with AML secreted tissue activator, cells from 45 patients secreted the urokinase-type enzyme, and cells from 5 patients secreted a mixture of urokinase- and the tissue-type activator. Cells from 7 patients with acute myeloblastic leukemia secreted too little enzyme for the activator to be identified with certainty (Table 1).

Cells from 32 patients with chronic myeloid leukemia were studied. Of these, 13 secreted urokinase, 12 secreted tissue activator, 5 secreted a mixture of urokinase and the tissue-type activator, and cells from the remaining 2 patients secreted too little enzyme to identify.

Neutrophils isolated from 23 normal subjects invariably released only the urokinase-type enzyme.

It is of interest to note that, in two cases, a plasminogen activator was observed that migrated electrophoretically as a single enzyme band with a molecular weight corresponding to that of urokinase. It was, however, unaffected by antibody to urokinase and completely inhibited by antibody to the tissue activator (Fig. 7).

Therapeutic Correlations

Cells from 72 patients with AML were studied with a view to defining the molecular species of plasminogen activator they released and correlating these with responses to therapy. The combined results are presented in Table 1, from which it can be seen that, taken overall, cells from 15 cases released tissue activator and those from 45 cases released the urokinase-type enzyme. In 5 instances, a mixture of tissue activators and urokinase was released, and in 7 cases the amount of enzyme released was too low for reliable identification.

This general tendency for approximately 20% of AML patients to have cells that released tissue activator was apparent in each of the three major therapeutic subdivisions. Thus, blasts from 4/14 patients who received palliative therapy, 3/16 patients who were treated with standard combination chemotherapy but who died before evaluation could be completed, and 8/42 patients in whom results of therapy could be assessed released tissue activator.

If, however, one considers only the 42 patients in whom the therapeutic response could be determined, it can be seen that in 81% of (25/31) patients whose cells released the urokinase-type enzyme, a remission was satisfactorily induced. In contradistinction, all 8 patients whose cells released tissue activator alone failed to enter remission.

In this limited series, therefore, there was a significant correlation (χ² = 17.8 p < 0.001) between the release of tissue activator alone and a poor response to the cytotoxic regimen that was used.

Since it has been reported by others that age and white blood cell (WBC) count at the time of presentation are adversely related to prognosis, patients whose cells released tissue activator and urokinase type enzymes were compared with respect to these parameters. Although differences were found, in no case were these statistically significant. Mean values ± SE of mean for patients whose cells released tissue activator were: age, 47 ± 3.9; total WBC, 33 ± 14.2. Corresponding values for those whose cells released urokinase were: age 37 ± 2.8; total WBC, 46 ± 9.1.

DISCUSSION

As previously observed with fibrinolytics released by other human cell types cultured in vitro, we have found that when fibrinolytic or caseinolytic activity was released by human peripheral blood leukocytes of the myeloid series, this was invariably plasminogen dependent. One is therefore justified in regarding these enzymes as plasminogen activators. This was true for both leukemic and normal cells and confirms the observations made by Granelli-Piperno et al. for normal human polymorphonuclear leukocytes.

The amount of plasminogen activator secreted by leukemic cells in culture varied widely and could not be correlated with the clinical severity of the disease. Although blasts isolated from the peripheral blood of patients with AML tended to release more plasminogen activator than did CML cells, there was a considerable overlap between these groups.

Using antibodies to urokinase and tissue activator in combination with electrophoretic analysis, we have shown that normal peripheral blood granulocytes released exclusively urokinase-like plasminogen activator, whereas leukemic cells secreted either a urokinase-like enzyme or a tissue-activator-like enzyme. The data therefore allow the tentative conclusion that the detection of tissue plasminogen activator release by peripheral blood cells is indicative of the leukemic state.

In most cases, cells isolated from any given leukemic blood sample generally secreted only one molecular species of plasminogen activator. This observation merits additional comment in the following three respects.
First, patients with AML whose peripheral blood leukocytes released exclusively activator of the tissue type tended to have a worse prognosis in terms of their susceptibility to induction of remission than did patients whose cells released enzyme of the urokinase type. If more extensive studies confirm this correlation, the molecular species of plasminogen activator released by AML blast cells may serve as a diagnostic marker of relevance to the management of this disease.

Second, we have isolated cells from 5 patients with AML that released both the urokinase-type and the tissue-type of plasminogen activator. In these cases, the cells appeared to be exclusively blastic and of leukemic type, suggesting that certain leukemic clones may develop that synthesize and release both forms of plasminogen activators. The report of a cloned cell line that produces two immunochemically unrelated forms of plasminogen activators provides precedent for this suggestion.

Third, our previous experience with plasminogen activators released by human cells has led us to believe that the electrophoretic finding of a 60,000 mol wt plasminogen activator, and the absence of a 70,000 mol wt enzyme in the same track, was sufficient to identify the enzyme as being of the urokinase type. The results we have obtained with two leukemic cell isolates have provided the exception that invalidates the generality of this assumption. We can now say with confidence that it is possible to observe tissue plasminogen activators that cannot be distinguished by electrophoresis from the urokinase type of enzyme. Immunochemical procedures are therefore essential for the correct identification of these enzymes.

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The Effects of Dexamethasone and Tetradecanoyl Phorbol Acetate on Plasminogen Activator Release by Human Acute Myeloid Leukemia Cells

By E. Lynnette Wilson, Peter Jacobs, and Eugene B. Dowdle

This investigation was undertaken to examine the extent to which leukemic cell functions are susceptible to regulation in vitro and to investigate their heterogeneity in this regard. Since plasminogen activator release is known to be a modulatable cellular function that can be influenced by antinflammatory steroids and tetradecanoyl phorbol acetate (TPA), the effect of these two compounds on the secretion of urokinase- or tissue-type enzymes by leukemic cells was studied. The release of both enzyme species could be stimulated or suppressed by these substances by mechanisms that were inhibited by actinomycin-D and hence required transcription of new mRNA. Plasminogen activator release by cells from 41/45 patients with AML was either stimulated or inhibited by $10^{-7}$M dexamethasone, implying that most AML cells possess glucocorticoid receptors. In 26/45 cases, the enzyme was inhibited by this steroid to less than 25% of control values. Pronounced inhibition of this degree was not encountered with normal polymorphonuclear leukocytes. Plasminogen activator secretion by AML cells was profoundly inhibited in 20/41 cases by 1 ng/ml TPA and stimulated in 8/41 cases. Leukemic blasts varied considerably in their response to dexamethasone and TPA. Plasminogen activator release should prove a sensitive means of monitoring the responses of AML cells to biologically active compounds.

Peripheral blood leukocytes from patients with myeloid leukemia release serine proteases that function as activators of the plasma zymogen, plasminogen. In a recent study of this phenomenon, we have observed that leukemic cells derived from different patients were dissimilar with respect to plasminogen activator release, both qualitatively in terms of the rate of enzyme secreted per cell, and quantitatively in terms of the molecular species of plasminogen activator that was secreted. Leukemic cells secreted either the urokinase-type or the tissue-type plasminogen activator. In acute myeloid leukemia (AML), cellular tissue-type plasminogen activator release was associated with an unfavorable prognosis for induction of remission.

In most other systems that have been studied, synthesis and release of plasminogen activators have been found to be inducible cellular functions that can be modulated by hormones, retinoids, and other compounds that affect expression of the malignant phenotype. It therefore seemed possible that an in vitro study of the effects of pharmacologically active substances on the synthesis of plasminogen activators by leukemic cells would provide a quantitative means of estimating the susceptibility of these cells to genetic modulation. Such information would contribute to the knowledge of the biology of the leukemic cell and might provide a rational basis for the classification and treatment of leukemias.

In this article we record our observations on the effects of the antiinflammatory steroid, dexamethasone, and the tumor promoter, tetradecanoyl phorbol acetate (TPA), on plasminogen activator release by AML cells incubated in vitro. These two compounds were chosen for their ability to influence plasminogen activator release in other systems. Furthermore, TPA has been shown to induce differentiation in the human promyelocytic cell line HL-60 and in cells from patients with AML.

MATERIALS AND METHODS

Subjects

Blood samples were obtained from 15 healthy laboratory workers and 45 patients with AML. All diagnoses of leukemia were based on histologic and histochemical examination of peripheral blood and bone marrow specimens. Romanowsky-stained preparations, cytochemical stains, and ultrastructural features were used to classify specimens according to the French-American-British recommendations.

Cells

Blood was taken by sterile venipuncture into tubes containing preservative-free heparin (Thrombolykin, Organon Teknika, Holland) to give a final concentration of 5 U/ml. Cells were fractionated by dilution with RPMI 1640 medium and centrifugation on a layer of Ficoll-Hypaque. Leukemic blast cells were harvested from the plasma-ficoll interface and freed of contaminating erythrocytes by incubation for 5 min in 0.83% ammonium chloride (pH 7.4). Polymorphonuclear leukocytes (PMN) were isolated from the blood of normal individuals as described by Granelli-Piperno et al.

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Experimental Protocol

Cells were washed once by centrifugation and suspension in RPMI 1640 and were resuspended in RPMI 1640 containing 3% fetal calf serum (FCS) to give $4 \times 10^6$ cells/ml. One-milliliter samples of this suspension were added to 35-mm Falcon plastic Petri dishes, and dexamethasone or TPA were added to give concentrations covering the ranges $0-10^{-5} M$ and $0-10$ ng/ml, respectively. Dexamethasone (Sigma Chemical Co., St. Louis, Mo.) and TPA (Dr. P. Borcher, Minn.) were kept as stock solutions in absolute ethanol at $10^{-7} M$ and 100 ng/ml, respectively. These were diluted in RPMI 1640 containing 3% FCS so that the addition of 10 μl to each 1 ml of medium gave the desired final concentration. The dishes were incubated for 24 hr at 37°C in a humid atmosphere of 5% CO₂ in air. At the end of this period, the medium was harvested by centrifugation and replaced with fresh medium containing compounds at the same concentration as before. At the end of this second period, the medium was again collected by centrifugation. The medium samples, referred to as harvest fluids, were stored at −80°C for analysis of plasminogen activator activity.

Cells were inspected under phase contrast microscopy after 24 and 48 hr of in vitro incubation. Cellular viability was assessed by Trypan blue exclusion.

Plasminogen Activator Assay

Plasminogen activator activity in harvest fluids was assayed by measuring plasminogen-dependent release of soluble radioactive fibrin degradation products from insoluble ¹²⁵I-fibrin-coated multiwell tissue culture plates (Linbro, Cat. no. FB-16-24, Flow Labs). This was done as previously described,²² save for the addition of 4 μg of plasminogen per well instead of 2 μg. Results were calculated in terms of urokinase units by reference to urokinase standards assayed simultaneously.

Immunohistochemical Analysis of Plasminogen Activators

Molecular species of plasminogen activators present in harvest fluids released by normal and leukemic cells were identified as urokinase-type or tissue-type using specific inhibitory antibodies to these enzymes. Harvest fluid samples were incubated for 1 hr at 4°C with serial twofold dilutions of purified rabbit antibody and assayed for residual activity using the ¹²⁵I-fibrin assay. All procedures have previously been described in detail.²²

Effect of Compounds on DNA Synthesis

Cells ($4 \times 10^6$) were suspended in 1 ml of medium containing dexamethasone or TPA at the required concentration and incubated for 18 hr before adding ³H-thymidine to give a final concentration of 5 μCi (1 μg)/ml. After a further 8 hr of incubation, 1 ml of ice-cold 10% trichloroacetic acid was added to each culture dish and dishes were set at 4°C for 30 min. The precipitate was then collected on a Whatman GFC filter and washed with 30 ml of 5% ice-cold trichloroacetic acid. The filter was dried and counted in Liquifluor-toluene in a Packard liquid scintillation spectrometer.

RESULTS

Normal granulocytes and cells from patients with AML differed markedly in their ability to survive in culture. At the end of 48 hr, at least 85% of leukemic cells were still viable, whereas only approximately 60% of normal granulocytes survived for 24 hr, and by 48 hr, this figure dropped to 30%. The limited capacity for in vitro survival shown by granulocytes has necessitated our confining our observations on these cells to the first 24-hr period of incubation. Since maximal effects of TPA and dexamethasone on plasminogen activator release by AML cells were observed during the second 24-hr period of exposure to these compounds, the results obtained during this period are presented.

We present the effects of TPA or dexamethasone on plasminogen activator release in terms of suppression or stimulation relative to control values observed in the absence of these compounds. For the sake of brevity and descriptive convenience, we have arbitrarily graded these effects as follows: pronounced suppression—less than 25% of control value; moderate suppression—25%-80% of control value; insignificant effect—80%-140% of control value; stimulation—greater than 140% of control.

Effects of TPA and Dexamethasone on Cellular Morphology and Viability

During the first 48 h of in vitro culture, TPA at 0.1 ng/ml, 1 ng/ml, or 10 ng/ml had no adverse effects on

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![Graph](image_url)  
Fig. 1. The effect of $10^{-7} M$ dexamethasone on plasminogen activator secretion by AML cells and PMNs. Each point is derived from a different subject and represents the average of the results for duplicate cultures expressed as a percentage of the average of results from triplicate control cultures to which steroid was not added. Urokinase-like enzyme (●), tissue activator-like enzyme (○), mixed urokinase-like and tissue activator-like enzyme (◆).
the viability of AML cells. In contrast, TPA at 1 ng/ml was moderately toxic to normal granulocytes, and at 10 ng/ml it was uniformly lethal within 24 hr.

As reported by Pegoraro et al., TPA induced a striking change in the morphology and adherence of AML cells in culture. At 10 ng/ml, TPA caused the cells to adhere to the plastic surface as clusters or as spread macrophage-like cells. Adherence was also frequently observed at 1 ng/ml but was usually not noted at 0.1 ng/ml.

Dexamethasone had no effect on the morphology of normal or leukemic cells. At $10^{-6}M$, this hormone occasionally caused viability to decrease during the first 48 hr of in vitro culture; at $10^{-7}M$ this effect was not seen.

**Plasminogen Activator Release by Normal or Leukemic Cells**

Enzyme secretion by leukemic cells of the 45 patients included in this study ranged from 0.001 to 22.1 urokinase units/10^6 cells/24 hr. Of these cells, 32 secreted plasminogen activator of the urokinase-type and 11 secreted plasminogen activator of the tissue-type. In two cases, both enzymes could be detected.

Neutrophils from normal subjects released from 0.006 to 0.3 urokinase units/10^6 cells/24 hr. In all cases this enzyme was of the urokinase-type. These results have previously been reported. 1

**The Effects of Dexamethasone on Cellular Plasminogen Activator Release**

Dexamethasone was added to cultures of normal granulocytes and AML cells to give final concentrations ranging from 0 to $10^{-6}M$, and harvest fluids were assayed for plasminogen activator content. The results observed when cultures were treated with $10^{-7}M$ dexamethasone are presented in Fig. 1, where each point represents the average of the results for duplicate cultures expressed as a percentage of the average of results from triplicate control cultures to which steroid was not added.

In 12/13 cultures of normal PMN, $10^{-7}M$ dexamethasone caused moderate suppression of plasminogen activator release. In the remaining case, the steroid had little effect.

The effects of dexamethasone on AML cells were more complex and in many cases more striking. Dexamethasone at $10^{-7}M$ either stimulated, had relatively little effect, or inhibited plasminogen activator release. Typical examples of these 3 responses are shown in Fig. 2.

In most cases, the effects of dexamethasone were time-dependent (being more obvious after 48 hr than they were after 24 hr) and concentration-dependent, being maximal or very nearly so at $10^{-7}M$. These
relationships are illustrated in Fig. 3. Two unusual responses were observed in which the steroid caused pronounced inhibition of plasminogen activator release at $10^{-4} M$, whereas it stimulated at $10^{-7} M$.

There was a tendency for the release of the tissue-type plasminogen activator to be inhibited to a greater extent than release of urokinase but this was not statistically insignificant. In 6/45 cases, $10^{-7} M$ dexamethasone stimulated plasminogen activator release, and in 35/45 cases, significant suppression was observed. Dexamethasone had an insignificant effect on cells from the remaining 4 cases (Fig. 1). In 26/45 cases, plasminogen activator release by leukemic cells was inhibited to less than 25% of control values. Pronounced inhibition of this degree was not encountered with any of the preparations of normal PMN studied at any of the concentrations of dexamethasone.

Results obtained with $10^{-8}$ and $10^{-4} M$ dexamethasone were similar to those obtained with $10^{-7} M$, whereas $10^{-8} M$ dexamethasone had significantly less effect on plasminogen activator release.

The effect of dexamethasone on plasminogen activator secretion could be prevented if actinomycin-D (1 µg/ml) was added to the cultures together with the dexamethasone, indicating that transcription of new mRNA was required for its inhibitory or stimulatory effect to manifest itself.

**The Effects of TPA on Plasminogen Activator Secretion**

Leukemic and normal cells were treated with TPA at concentrations of 0.1, 1, and 10 ng/ml and harvest

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**Fig. 3.** AML cells were treated with the indicated concentrations of dexamethasone for two consecutive 24-hr periods as described in Materials and Methods. Harvest fluids from untreated (Con) and steroid-treated cells were then assayed for plasminogen activator activity.

**Fig. 4.** The effect of 1 ng/ml TPA on plasminogen activator secretion by AML cells and PMN. Each point is derived from a different subject and represents the average of the results for duplicate cultures expressed as a percentage of the average of the results from triplicate control cultures to which TPA was not added. Each point represents results obtained from a separate individual. Urokinase-like enzyme (○); tissue activator-like enzyme (●); mixed urokinase-like and tissue activator-like enzymes (□).
fluids were collected for plasminogen activator assay as described for steroid-treated cells. The results were compared with those obtained with the same cells were incubated under identical conditions without TPA. The data obtained with 1 ng/ml TPA are presented in Fig. 4, where each point represents the average of the results for duplicate cultures expressed as a percentage of the average of results from triplicate control cultures to which TPA was not added.

When added to normal neutrophils, TPA at 1 ng/ml caused a moderate suppression of plasminogen activator release in 2/15 cases; in 5/15 cases it had no significant effect, and in 8 cases it stimulated between 2 and 10-fold.

The effects of TPA on AML cells varied considerably. When added at 1 ng/ml, the compound caused profound inhibition of enzyme release in 20/41 cases and stimulated in 8/41 cases. Cells that released urokinase showed a tendency to be inhibited by TPA, whereas cells that synthesized tissue activator were stimulated. This difference was not significant when examined by the Mann-Whitney U test. In all cases where inhibition was seen, viable cell counts excluded the trivial explanation of a cytotoxic effect of the TPA.

Results obtained with 10 ng/ml were essentially similar to those obtained with 1 ng/ml, while 0.1 ng/ml had a less marked effect.

The effects of TPA on plasminogen activator release could be prevented by actinomycin-D in a similar manner to that described previously for dexamethasone.

Combined Effects of Dexamethasone and TPA on Plasminogen Activator Release and DNA Synthesis

Although it had been established that, as used in our experiments, neither TPA nor dexamethasone diminished the viability of leukemic cells, we felt it important to exclude cytotoxicity as an explanation for our observations in a more definitive experiment. This was accomplished by the simultaneous measurement of DNA synthesis and plasminogen activator release as a function of dexamethasone and TPA concentration in the same leukemic cells. Results of such an experiment showed that dexamethasone caused inhibition of plasminogen activator release yet had no effect on DNA synthesis. Conversely, TPA at 10 and 1 ng/ml stimulated plasminogen activator release, yet inhibited DNA synthesis.

DISCUSSION

In this article we have shown that dexamethasone and TPA modulated the rate of release of plasminogen activators by normal and leukemic cells cultured in vitro. These effects were not due to nonspecific toxic phenomena and required the transcription and translation of new mRNA for their manifestation.

Although, as observed by Granelli-Piperno et al., dexamethasone suppressed and TPA tended to stimulate plasminogen activator release by normal granulocytes, these effects were generally less marked than those observed when AML cells were treated with these compounds at the same concentrations. Differences between AML cells and normal PMN in these respects may have been due to differences in their neoplastic status or in the extent to which they had differentiated. Normal bone marrow myeloblasts would have been a preferable control cell population to have used and such studies are currently in progress. Furthermore, PMN cells survived poorly in culture, so that it was difficult to design experimental protocols that could be used to draw valid comparisons between AML cells and normal granulocytes. For these reasons we do not wish to suggest that differences between normal granulocytes and AML cells that we have observed necessarily identify differences between normal and leukemic cells.

By most clinical and laboratory criteria, the leukemias comprise a group of well defined disorders. Within each diagnostic category, however, one frequently observes patients whose disease differs strikingly in its presentation, progress, and response to therapy. The results in this article provide further examples of this phenotypic diversity.

Plasminogen activator release by AML cells incubated in vitro differed between patients both in terms of the type and the amount of enzyme secreted and in terms of the extent to which this secretion could be modulated by dexamethasone or TPA. The regulation of plasminogen activator synthesis and release has proved to be a genetically controlled cellular function in all cells studied to date. Our data provide good reason to believe that the same holds true for AML cells and that the phenotype of each myeloid leukemic cell clone is uniquely governed by the action of genes that vary in the degree to which their expression can be modulated. Sufficient data are not yet available to allow significant correlations to be drawn between modulation of plasminogen activator release, other criteria of cellular differentiation and clinical features of this disease.

Since cells from 41/45 patients with AML showed a significant change in the rate of plasminogen activator secretion in response to 10⁻³ M or 10⁻⁴ M dexamethasone, one might infer that the great majority of AML cells possess glucocorticoid receptors. Using techniques that measure binding of radioactive steroid, Lippman et al. and Gailani et al. found that 20%–30% of disrupted AML cell specimens possessed glucocorticoid receptors. However, when intact cells were used for receptor measurement, leukemia blasts from all patients were found to contain glucocorticoid
receptors. Our results tend to confirm the need for intact cells for receptor measurements. The most sensitive and informative assay may be the indirect enzymatic one that we have used, in which the effects of steroid-receptor engagement are amplified many-fold by effects on cellular plasminogen activator release.

Most studies of the effects of dexamethasone on plasminogen activator synthesis have reported inhibition of enzyme release by this steroid. Roblin has recently suggested that the synthesis of the urokinase-type enzyme is inhibitable by dexamethasone, whereas that of tissue plasminogen activator is not. Our observations with leukemic cells show that this is not a general rule. The release of both tissue-type and urokinase-type plasminogen activators were susceptible to the effects of dexamethasone, and in these cells, both enzyme species could be inhibited or stimulated by this steroid (Figs. 1 and 2). Therefore, it is the cell type rather than the species of enzyme that is the final determinant of dexamethasone responsiveness and, unlike other cell types studied, AML cells may show stimulation of plasminogen activator secretion in response to this glucocorticoid.

Although TPA is best known as a promoter of carcinogenesis and for its ability to enhance expression of the transformed phenotype, it has been shown to induce differentiation in leukemic cells. Similarly, TPA usually induces the synthesis of plasminogen activator when added to other cells cultured in vitro, yet as shown by our studies, this compound usually inhibits release of this enzyme when applied to leukemic cells (Fig. 4). In these two respects, therefore, AML cells respond to TPA in an anomalous fashion. The reason for this and its significance must await further study.

In conclusion, we feel it appropriate to emphasize the usefulness of the measurement of plasminogen activator synthesis as a sensitive means of monitoring cellular responses to biologically active compounds. The implications of this conclusion for rational approaches to the management of AML are obvious. It is hoped that further studies will justify this conclusion and will establish the characterization of plasminogen activator release as a useful procedure for predicting prognosis and optimal therapeutic options in this disease.

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EDITORIAL

Bone Marrow Transplantation

Bone marrow transplantation has emerged as the preferred form of treatment for certain clinical situations exemplified by immunodeficiency diseases, and for patients with severe acute aplastic anaemia. The place of this procedure in the treatment of individuals with acute leukaemia is less clearly defined, although a small number of long-term survivors has established it as justifiable when conventional methods have failed. In view of the steady progress in understanding the principles of bone marrow transplantation, and the availability of this service in the Republic, it is timely to review the current status of this form of management.

The transfer of marrow from one individual to another is not new, and its feasibility was demonstrated in animal studies more than a quarter of a century ago. Transplantation in humans was initially abortive, and the poor results obtained in these early studies restricted its clinical application. However, from 1970 the principles of tissue typing have been better appreciated, and this knowledge, coupled with advances in immunosuppressive therapy, has led to a number of long survivals, so that bone marrow transplantation may now be regarded as both practical and realistic. Nevertheless, a number of problems remain unresolved, and it is important to place in perspective what can be achieved with this procedure, in order to define current indications and to identify those diseases which require further study before transplantation can be included among the therapeutic options.

In severe combined immunodeficiency disease, morbidity and mortality are high, and transplantation of bone marrow from a suitably matched sibling is the treatment of choice. In this situation it is noteworthy that haematopoietic function is usually intact, and only immunological reconstitution is necessary. Accordingly, multiple small infusions of marrow, or even of fetal liver, may be effective in reversing the lesion. The severity of the immunological defect limits capacity for rejection, making engraftment relatively simple, even when minor histocompatibility differences exist and unrelated donors are used. However, graft-versus-host disease remains an unpredictable and formidable problem.

In bone marrow hypoplasia, results have been clouded by the lack of clear definition of the indications. However, criteria are now established for the selection of individuals who will have a significantly improved survival compared with that of matched patients who receive only supportive and conservative therapy. These include platelet counts of less than 20 000/μl, granulocytes less than 500/μl and reticulocytes of less than 1% when corrected for anaemia, together with a loss of haematopoietic cells from a bone marrow trephine biopsy specimen.

The fact that the majority of patients with severe bone marrow hypoplasia can have their marrow successfully reconstituted in this way is evidence in favour of the defect's being in the stem cell rather than in the micro-environment. However, some patients have rejected the graft, but have nevertheless recovered, with regrowth of autologous marrow. The latter findings support the concept that, at least in some individuals, severe acute aplasia may involve a lesion other than that of the stem cell.

In aplastic anaemia, the longest period of survival after a transplant from a monogygotic twin is 16 years, while the 3 patients treated in Seattle are presently alive and well. The same series, 31 of the 73 HLA-matched patients who received transplants from siblings are presently alive, the longest survival being 5 years. Essentially similar data have been reported by the international co-operative study group of which the investigators from Cape Town are members.

Against this background is important to note that such patients, immediately upon diagnosis, should be transferred to a transplantation centre so that they can be evaluated for what is currently considered the preferred form of treatment. Any delays in this diagnosis have been established, in anticipation of spontaneous resolution, and particularly when blood is administered, may compromise the success of later transplantation attempts. Conservative treatment should be reserved for patients with severe acute aplastic anaemia, in whom transplantation is, for one reason or another, not feasible.

In patients eligible for transplantation, success is statistically related to the adequacy of the number of donor cells infused and the absence of prior antigen sensitization. Second transplantsations from the same or an alternative donor can further increase the chance of success.

A second and unique immunological problem may arise once the graft has become established. Thus, donor lymphocytes recognize the recipient's antigens as foreign and this gives rise to a variable clinical pattern of graft-versus-host disease, with the burden of the damage being borne by the skin, the gastro-intestinal tract and the liver. This phenomenon will occur in approximately two-thirds of patients with HLA identity and no reactivity in the mixed lymphocyte reaction, suggesting the importance of other less clearly defined histocompatibility antigens. The precise genetic basis for this phenomenon is presently controversial, but its severity is clearly correlated with sex mismatch of the donor and the presence of refractoriness to random donor platelets at the time of transplantation.

Aplastic anaemia without available donor poses a special problem. In the first instance patients with some residual haematopoiesis, selected on ferrokinetic studies and stem cell growth patterns in vitro, may
benefit from treatment with lithium or etiocholanone. The anabolic androgens are unlikely to be of benefit in severe acute aplastic anaemia but, in contrast, some response may be achieved when hypoplasia of lesser degrees is present. However, those forms of treatment need to be critically evaluated against the possibility that immunosuppressive therapy, with or without marrow infusion, may result in remission. Furthermore, if the macrophage is centrally involved in the genesis of graft-versus-host disease, then selective attacks upon those cells with liposomes or platelets loaded with cytotoxic agents may offer a realistic approach to control of this immunological entity, which remains the major limiting factor in the use of marginally mismatched grafts.

The role of bone marrow transplantation in cases of acute leukaemia is undergoing critical review. Initially, the procedure was reserved for patients who had failed to achieve complete remission or who had recurrent disease. The poor results achieved with this approach initiated attempts to reduce the leukaemic body burden of patients before conditioning them for transplantation. While there is a small number of long-term survivors of these programmes, the median survival is not markedly different from that which can be achieved with modern chemotherapy and immunotherapy programmes. Analysis of recent information has identified three distinct periods following transplantation. The first 120 days are characterized by high patient mortality from advanced disease, graft-versus-host disease and associated infection, while recurrent leukaemia is a relatively insignificant problem. In a second period, from 120 days to 2 years, recurrent leukaemia causes additional mortality. Approximately 15% of the original patient group are now in a third period which has extended to 64 years, and in which leukaemia has not recurred. It would therefore be reasonable to conclude that bone marrow transplantation is curative for some patients, even, apparently, in the end-stage of the disease.

Clearly, bone marrow transplantation has an important role to play in the management of the patient with leukaemia, and the next step must be to define the most appropriate way of realizing the potential of this procedure to prolong the quantity and quality of life in patients afflicted with this disease. The fact that long survival is possible, although recurrent leukaemia remains a problem, suggests that effort should now be directed at evaluating bone marrow transplantation in the first complete remission rather than after relapse. One additional modification which must be considered is the storage of bone marrow collected at this time in anticipation of autograft support in the event of subsequent relapse. There are good reasons for serious consideration of this approach. Firstly, treatment will be given at a time when the body burden of tumour cell is low, and the selection of these patients should take into account all laboratory studies designed to recognize minimal residual disease. Secondly, the patient will be in good condition and therefore better able to tolerate the transplantation regimen. Thirdly, and importantly, the leukaemic clone will not have become resistant to therapeutic modalities. It may therefore be concluded that bone marrow transplantation from a monozygotic twin or from an HLA identical and MLC non-reactive sibling is the treatment of choice for patients with severe combined immunodeficiency disease or acute aplastic anaemia. In these situations the prognosis is so poor, and alternative approaches so unsatisfactory that, in the absence of a compatible sibling, there is a clear need for the extension of donor matching to a national scale, or for the development of collaboration with other countries. The question of acute leukaemia poses two problems. The first is the management of patients who have drug-resistant disease, or who have relapsed, and whose prognosis is poor. Since some of these individuals can be salvaged by transplantation, and have long disease-free periods, this approach remains a rational form of management. However, in view of the generally unsatisfactory median survival of the latter group of patients, it may be preferable to use this valuable resource in those who are achieving their first complete remission. Finally, advances in the immunobiology of bone marrow transplantation are such that it should soon extend to lethal genetic disorders of other marrow elements such as homoygous thalassaemia, sickle-cell disease and qualitative defects in white cells.

P. Jacobs

THE PROFESSIONAL NURSE IN THE CELL SUPPORT UNIT

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THE NURSE — A CENTRAL FIGURE

The well-trained nurse is the central figure in the efficient operation of the Cell Support Unit. This individual is responsible for the safety of those undergoing the procedures, whether they be volunteer donors or patients and has as a prerequisite thorough training and competence in intensive nursing care.

To this basic requirement must be added a minimum of three months in an accredited training centre to become familiar with the operation of the cell separator in order that malfunction of the instrument and complications arising in donor or patient may be recognised immediately and appropriate corrective steps taken. This new breed of professional nurse reflects in part the introduction of sophisticated technology and in part the increasing role played by nurses at the forefront of health care.

THE UNIT

The Cell Support Unit consists of expensive sophisticated machines called blood fraction separators. These may be of different types but the one used at Groote Schuur Hospital (figure 1) is the NCI-IBM 2997 model which uses disposable pre-sterilised plastic tubing and a separation band (figure 2) which is assembled, inserted into the machine and all air flushed out with physiological saline. The machine function, integrity of the tubing, and the alarm systems are tested before the subject is started on the procedure.

Each cell separator is operated by one full-time professional nurse who never leaves her station at any stage during the procedure. The machine can be used for different purposes including white cell collection, known as leucopheresis, platelet harvesting, known as plateletapheresis, plasma exchange or plasmapheresis, and red cell exchange. The different techniques are collectively known as apheresis.

The Cell Support Unit is ideally an integral part of the Department of Haematology, where it fulfils the function of providing white cells and platelets for the leukaemia and transplantation programme, collections of haematopoietic stem cells for bone marrow grafting, and a wide range of emergency procedures not only for haematology patients but for those in the remainder of the hospital.
Fig. 1 Blood fraction separator.

These include replacement of abnormal red cells in patients with sickle cell disease during crisis, plasma exchange for life-threatening antibody-mediated diseases such as myasthenia gravis and Goodpasture syndrome, or removal of immune complexes in rapidly progressive glomerulonephritis.

After the introduction and development of separators in university departments, they are being slowly introduced into blood transfusion services for more efficient collection of platelets; here as well, the professional nurse should be in control of the procedure.

APHERESIS TECHNIQUES

The technique is relatively simple. Intravenous cannulae are introduced into the brachial vein in the forearm. Blood enters the specially designed band in the machine where it is separated into components based on differences in their specific gravities by means of circumferential centrifugation. Any of the components can then be selectively removed by means of roller pumps and the remaining blood returned to donor or patient by intravenous infusion into the opposite arm. In exchange procedures, large volumes of red cells or plasma can be separated and discarded and blood volume and composition retained by appropriate infusion into the return line.

Many safety devices are incorporated into the equipment to minimise hazards to the subject arising from instrument dysfunction. No technology, however, can safely be left to operate without continuous supervision by a highly trained and thoroughly experienced nursing sister.

Fig. 2 Separation band.
Leucopheresis

Leucopheresis is the collection of white cells and with appropriate adjustment in technique this may be largely lymphocytes or, more usually, predominantly granulocytes.

Lymphocyte depletion may be used as a form of immunosuppressive therapy and is being investigated in the treatment of autoimmune and immunologically-mediated diseases. Granulocyte transfusion plays a vital role in patients with neutropenia, as seen in severe acute aplastic anaemia, following bone marrow transplantation, and after cytotoxic chemotherapy in leukaemic patients.

In each of these situations the peripheral blood granulocyte count should be less than 0.5 × 10⁹/l, the patient to have a sustained fever greater than 38.5°C, and having failed to respond to adequate courses of appropriate intravenous antibiotic therapy for 48 hours. Once granulocyte transfusions are commenced, they are continued until the count is greater than 0.5 × 10⁹/l, the infection controlled, and the temperature normal for 48 hours.

The procedure time for white cell collection is approximately two hours and depends upon the donor's white cell count. It is useful to administer 48 mg of methylprednisolone six to eight hours before commencing the collection to raise the white cell count. Under these circumstances, a 250 ml volume will contain between 2 and 4 × 10⁹ leukocytes which are morphologically and functionally normal.

During the procedure a concentrated citrate solution is infused as an anticoagulant in the ratio of 13:1 to the whole blood to ensure that clotting does not occur once blood is in the machine. A sedimenting agent in the form of 500 ml of hydroxyethyl starch is also added to the blood in the separator to improve granulocyte separation. The donors are always ABO and Rh group compatible with the recipients.

Plateletpheresis

Plateletpheresis is undertaken for bleeding when the platelet count is below 20 × 10⁹/l, particularly following chemotherapy for cancer. It is important that donors do not take any tablets containing aspirin or other antiplatelet drugs prior to donation since, although the numbers may be normal, their function may be suboptimal.

A similar situation is found in patients with myeloproliferative syndrome and when such individuals require surgery additional platelets may be needed despite normal numbers.

The procedure time is ninety minutes to collect 250 ml of plasma containing between 3 and 5 × 10¹¹ platelets. It is useful, following platelet infusion, to monitor the rise in platelet count which should be approximately 50 × 10⁹/l/m² for a single such pack and indications for further infusions can be gauged by documenting platelet survival. The latter measurement is simply carried out by twice daily platelet counts.

Plasmapheresis

Plasmapheresis refers to the separation and removal of plasma and its replacement with an appropriate fluid which may be either fresh, frozen plasma, fractionated serum, or plasmalyte B and albumin. A practical exchange is approximately four litres but will vary with the individual's plasma volume.

Flow rates between 30 and 50 ml/minute are achieved using acid citrate dextrose as an anticoagulant at a ratio of approximately 1:11 with the blood. Specimens are collected before and after the procedure to monitor the white cell count differential, platelet count, biochemical profile, and changes in clotting factor. Serum samples are stored to measure the level of the product removed, such as cholesterol, antibodies or immunoglobulin.

A wide variety of indications exist for plasma exchange. Firstly, the hyperviscosity syndromes, as in Waldenström's macroglobulinaemia, in multiple myeloma, and in cryoglobulinaemia where abnormal proteins are precipitated in the cold.

Secondly are antibody-related diseases where the procedure is carried out in conjunction with immunosuppressive therapy using prednisone, cyclophosphamide, or azathioprine and aimed at removing the antibody giving rise to the disease. Examples would include Goodpasture syndrome where renal failure and haemoptysis characterise the clinical syndrome, myasthenia gravis where weakness and paralysis are prominent clinical findings, and less frequently in haemophilia associated with antibodies to factor VIII, in diabetes with anti-insulin antibodies, in rheus sensitisation where anti-D causes haemolytic disease of the newborn, and in both immune thrombocytopenia and haemolytic anaemia.

Similarly, immune complexes formed between foreign antigen and antibody may produce life-threatening symptoms in systemic lupus erythematosus and fulminating glomerulonephritis.

Thirdly there is a group of miscellaneous conditions where plasma exchange may be used to remove poisons or drugs taken in overdose, removal of biologically active substances in hypercholesterolaemia, in liver disease, porphyria, thyrotoxic crisis, and even to remove blood group antibodies where incompatibility exists between donor and recipient prior to bone marrow transplantation.

Other indications

Continuous-flow red cell exchange is an efficient and practical way of removing abnormal haemoglobin (HbS) in patients with sickle cell anaemia and replacing this with normal adult haemoglobin (HbA). This may be done prophylactically where patients require surgery or therapeutically when patients present with sickle cell crises.

In addition, therapeutic leucopheresis may be done where very high white cell counts may interfere with blood flow, as in acute and chronic leukaemia, while platelets may be removed by means of therapeutic plateletpheresis in individuals at risk from thrombotic episodes due to thrombocythaemia.

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ROLE OF THE NURSE

In all of these sophisticated and relatively complex techniques, the role played by the professional nurse cannot be overstated and falls into two broad groups. Firstly, the thorough competence with all aspects of machine operation including the recognition of hazards and complications associated with the procedures. Secondly, the important role of donor recruitment.

Machine operation and patient observation

Hazards may be associated with the machine. Thus, air embolism may result from incorrect priming, extracorporeal clamping may reflect insufficient anticoagulant, while haemolysis may be due to a failure to recognise abnormal pressure changes occurring in the circuit.

Anticoagulants, particularly the acid citrate dextrose solution used in the procedures, may lead to side effects including citrate toxicity in which reduction of ionized calcium, due to binding, produces symptoms. The latter may be slight with numbness and tingling in the lips and around the mouth or the extremities, while priapism may be embarrassing. Failure to immediately recognise and correct citrate overdosage may lead to more severe side effects such as nausea and vomiting, subternal chest pain with changes in the electrocardiograph and even cardiac arrest. While correction is easy and involves reduction in flow rate, failure to obtain immediate reversal may require the intravenous administration of 10 ml of 10% calcium gluconate over the course of 10 minutes.

The sedimenting agent, hydroxyethyl starch, may result in urticaria, skin irritation with no visible changes which may last for many days, or headache due to plasma expansion. The replacement fluid, particularly when this is fresh, frozen plasma, may cause allergic reactions, fever, chills, urticaria, and hypotension.

Finally, the individual undergoing the procedure may present difficulties because of poor venous access, or anxiety, usually during the first procedure. This can be overcome by a confident operator, reassurance to the patient, and careful step-by-step explanation.

Syncope or fainting may occur. Haematoma may occur at the site of intravenous cannulation, particularly in the hands of inexperienced or poor operators and, similarly, blood may infiltrate the return site.

In each of these situations it is imperative that the professional nurse be able to recognise and separate anxiety from changes in plasma calcium level due to citrate intoxication or dilutional effects. Only experience will help the nurse to recognise more severe reactions that may occur during the course of these procedures and which may correlate with the underlying disease.

It is completely unacceptable to have a sister nominally present or in charge of such a unit. The only arrangement appropriate for an academic institution is to have a senior member of the nursing faculty positively and directly in charge of all aspects of the procedure and responsible for the supervision and in-service training of her more junior staff.

Donor recruitment

Neither should the question of donor recruitment, which lies within the ambit of the professional nurse, be underestimated. While in many situations families of patients come forward as volunteer donors, the demands of a busy unit require considerable support from the community and this can be elicited most efficiently by word of mouth in which one donor brings friends. To be efficient, such a system implies a happy unit competently staffed and with which the donor panel clearly identifies. Additional sources of donor recruitment are the media including the press, radio, television and illustrated short talks to large firms and factories.

Donor selection itself is important since each individual establishes a personal relationship with the nursing staff of the Cell Support Unit. It is the moral responsibility of the staff to fully explain the procedure to all donors, including the use of drugs, and then to obtain fully informed consent.

Initial screening includes blood and rhesus grouping, screening for hepatitis, malaria, and venereal disease, and excluding underlying serious illness. Donor age is not critical, lying anywhere between 18 and 55 years. A suitably large panel, meticulous control of rotation and a philosophy never to store products but to collect components only as the specific need arises will mean that donors are used about once every four months for white cell collections but monthly for platelet donation. Following bone marrow grafting, the donor may undergo apheresis daily for five days to collect haematopoietic stem cells.

CONCLUSION

It is concluded that the professional nurse plays a vital role in the Cell Support Unit. The services range from collection of white cells and platelets through continuous-flow red cell, white cell, and platelet exchange to plasmapheresis. In each of the procedures sophisticated equipment with numerous fail-safe devices is used.

Nevertheless, it remains the cardinal principle that safety rests solely on the shoulders of the professional nurse in charge of the procedure. Only thorough familiarity with every aspect of the instrument and the procedure is compatible with patient safety and there is no excuse for leaving an instrument unattended at any stage of its operation. These prerequisites for donor and patient safety emphasise the everexpanding role of the professional nurse as an equal partner with the doctor in delivery of modern health care services.

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Review Article

Trephine biopsy of the bone marrow

A. R. BIRD, P. JACOBS

Techniques of trephine biopsy

Many methods of trephining the bone marrow have been described. The Vim-Silverman needle was the first device to be used regularly. This was followed by the introduction of the Westerman-Jensen needle, a modification of the Vim-Silverman needle, which is still used in many centres. The technique is excellently illustrated by Ellis et al. More recently, the Jamshidi needle was introduced and is now probably the most widely used device. Other coring techniques using the Border trephine, Gidlund's instrument, the Radner needle, the Gardner and the Sacker-Nordin needle have been described. Larger specimens may be obtained with Burkhardt and Notter-Labhart needles or by open surgical biopsy. These procedures are, however, time-consuming and therefore unsuitable if serial biopsies are required.

Our preference is to use the Jamshidi needle. The technique of coring is outlined by Jamshidi and Swaim and is well known to most haematologists. We have also had extensive experience with the Westerman-Jensen needle but now prefer the Jamshidi needle for the following reasons: (a) there is little disturbance in the architecture of the core obtained by the Jamshidi needle; (b) it requires less maintenance than the Westerman-Jensen needle since sharpening and replacement of cutting blades are avoided; (c) larger cores are obtained; and (d) it is our experience that patients tolerate trephining with the Jamshidi needle better than with the Westerman-Jensen needle.

Certain points, however, should be noted. Firstly, the cutting tip of the Jamshidi needle should be sharpened regularly and the tip should be kept acutely angled to reduce the incidence of 'lost cores'. Secondly, we have experienced difficulty in obtaining specimens from very osteoporotic patients using large-gauge Jamshidi needles. In these circumstances, the Westerman-Jensen needle is often preferable. Thirdly, the anatomy of the pelvis must obviously be considered when performing a trephine biopsy. The posterior iliac crest (the most convenient and probably the safest site for marrow biopsy) is usually easily palpable with the patient lying prone. The sacro-iliac joint lies perpendicularly below the posterior crest. The wing of the ilium slopes laterally and it is in this direction that the biopsy needle should be aimed.

We routinely perform marrow aspiration just prior to biopsy. We do not aspirate with the same needle but prefer to use the Klein or similar needle. As long as the needle is directed away from the site of the proposed biopsy, there is no distortion on the subsequent trephine sections.

It is well known that aspiration of marrow can cause sharp pain. Trephining of the marrow, with few exceptions, seems to cause less discomfort. Unallayed anxiety, inadequate local anaesthesia and poor technique are factors which may make the procedure unacceptable. Physicians inexperienced in the technique of trephine biopsy usually provide inadequate specimens and therefore the procedure has to be repeated. In our hospital, therefore, all patients requiring bone marrow examination are referred to the Haematology Department. The marrow aspirates and trephine biopsies are then performed by the residents.
Preparation of the specimen for examination

Fixation

Once an adequate core (> 2.0 cm) has been obtained, it is fixed for a minimum of 4 hours in Zenker’s fixative, with constant agitation. It is washed overnight to remove the dextranate and the following morning is placed in Formal/Decal solution for 4 hours to decalciﬁy the bone using a roller mixed to ensure even exposure of the core to solution. After washing for 15 minutes, it is then processed and embedded in Paraplast according to standard methods.

Poor-quality sections are often the result of one or more of three problems involving fixation: (i) inadequate time allowed for proper ﬁxation — we have run a number of trials to assess optimum ﬁxation time, and 4 hours is the absolute minimum using Zenker’s ﬁxative; (ii) using a Zenker’s stock solution older than 2 weeks; (iii) ﬁxative prepared too far in advance of the biopsy procedure; it should be prepared just prior to biopsy.

Preparation of sections

The preparation of good sections ensures accurate identiﬁcation of the haematopoietic cells. Ideally, serial sections should be examined. We recommend that at least three sections taken at different levels should be studied. If properly decalciﬁed and ﬁxed, sections can be cut at 4 µm. By using a methacrylate embedding process, sections as thin as 1 µm can be prepared.17 This allows for very accurate identiﬁcation of haematopoietic cells. The major disadvantage of this technique is the time required for preparation of the blocks, usually a minimum of 4 days.

Staining procedures

At least three sections from each core biopsy specimen should be stained with haematoxylin and eosin, and one section for reticulin. Romanovsky stains have been recommended2 but in our experience add little diagnostic information. Iron stores are best evaluated on the aspirate smears using Perl’s reaction, but if smears are not obtainable, sections may also be stained for assessment of iron stores. Loss of iron from the section, however, may occur during ﬁxation and we do not recommend core biopsies for the assessment of iron stores. Special stains for fungi or tubercle bacilli may be indicated in the presence of granulomas, although these stains, too, may be affected during the ﬁxation processes.

Indications for trephine biopsy

In the following section we outline what we consider to be absolute indications. In these situations, failure to do a trephine biopsy may result in a missed or delayed diagnosis.

Failure to aspirate marrow

Three failed aspirations from different puncture sites should be regarded as a ‘dry tap’. In these cases, material for cytological assessment may still be obtained by performing a touch preparation with the trephine biopsy.

Myeloproliferative syndrome (Fig. 1)

This is probably the commonest condition leading to failed aspiration. The appearance of the marrow is pathognomonic,14 although in the early cellular phase of polycythaemia vera the diagnosis may not be obvious. A stain for reticulin is mandatory, however, as even in polycythaemia vera the typical pattern of increased marrow reticulin is seen.

Metastatic neoplasia (Fig. 2)

‘Dry taps’ in patients with cancer metastatic to the marrow are not infrequent. A review of 74 marrow aspirates and trephine specimens obtained over a 2-year period (January 1973 – December 1974) from patients with non-haematological malignant disease showed the presence of metastatic tumour in 18 (24%). In 7 of these tumour was found in both the aspirate and the trephine specimen, while 5 were ‘dry taps’. In only 1, a child with metastatic retinoblastoma, was the aspirate positive in the face of a negative trephine specimen (A. R. Bird and P. Jacobs — unpublished observations).

Fig. 1. Silver stain showing dense parallel strands of reticulin in a patient who presented with pancytopenia and splenic enlargement. Myelofibrosis was diagnosed.

Fig. 2. Metastatic carcinoma with intense desmoplastic reaction and blood vessel invasion in a patient with fever and weight loss who was eventually found to have a 8.5 cm primary carcinoma of the proximal colon.

In a study by Savage et al.4 utilizing a Jamshidi needle for biopsy and aspiration, a correlation between biopsy and aspiration results was shown in 75% of cases positive for metastatic cancer. In only 1 instance was the aspirate positive and the trephine biopsy negative. The remaining discrepancies were all due to biopsy-positive, aspirate-negative specimens. Other studies also attest to the efficacy of the trephine biopsy in increasing the yield of positive specimens.11,38 Performing bilateral tre
phine biopsies further increases the yield of positive specimens.26
Garrett et al.,21 however, reviewed 291 patients with non-
haematological malignancies and found 39 positive for metastatic
tumour. The biopsy was positive in 3 patients with a negative
aspirate, and in 3 others the aspirate was positive with a negative
biopsy. They attributed their high rate of tumour detection in
the aspirate to a thorough initial screening by technologists; this
process, however, sometimes occupied several hours in scanning
many smears.

The yield of positive marrow specimens may be increased if
biopsies are performed at sites of local tenderness or in a
suspicuous area as depicted on the radiograph or bone scan.
However, needle biopsies can be performed safely only from the
anterior or posterior iliac crests, or occasionally from the verte-
bral spines. Negative radiographic studies or bone scans do not
mitigate against the performance of a marrow biopsy, as positive
marrow specimens may be obtained in the case of a negative
skeletal survey or scan.22

Concurrent aspiration and trephine biopsy from the posterior
iliac crest, bilaterally if possible and aided by radiographic stu-
dies and a bone scan, should therefore ensure the best possible
yield of positive specimens from the marrow in the diagnosis of
metastatic tumours.

Acute leukaemia
Although aspirate smears are indicated for the cytological
diagnosis of acute leukaemia, the marked increase in reticulin
sometimes seen in both acute lymphoblastic and acute myelo-
blastic variants may lead to a 'dry tap'. A recent study has shown
that the presence of this reticulin does not appear to be related to
the prognosis,23 although there has been a report to the con-
trary.24 Induction of remission in patients with increased mar-
row reticulin results in a distinct reduction in the reticulin.
Recurrence of increased reticulin may herald the onset of a
relapse of the leukaemia.25,26

In the follow-up of patients with acute leukaemia, and particu-
larly in the post-induction period of hypoplasia, marrow biopsy
allows for a more comprehensive assessment of marrow reserve
than the scanty particles obtained on aspiration alone.

Leukaemic reticulo-endotheliosis
This rare but well-described condition mainly affects adults,
and patients with pancytopenia and splenomegaly. The diag-
nosis is usually made from the peripheral blood smear where the
diagnostic 'hairy' cells are seen.27 These cells show a characteris-
tic positive acid phosphatase reaction which is resistant to tar-
rate pretreatment.28 Attempted marrow aspiration is invariably
non-productive and a marrow biopsy is required. Sections show
the characteristic infiltrate with a dense reticulin pattern.

Staging and follow-up of patients with
lymphoma
Hodgkin's disease (HD) (Fig. 3)

Marrow specimens positive for HD show disruption of the
normal marrow architecture by fibrosis, necrosis, and a cellular
infiltrate of lymphocytes, plasma cells, eosinophils and histio-
cyes, even in the absence of Reed-Sternberg cells. The pattern
of infiltration may be diffuse or focal.27 Marrow aspiration alone
is of limited value in the detection of marrow involvement by
HD. This is not unexpected, in view of the often focal nature of
the infiltrate and the high incidence of accompanying fibrosis. A
review of 53 trephine biopsies and aspirate smears from patients
with HD during a 2-year period from January 1973 to December
1974 revealed 19 positive specimens. Of these 4 were 'dry taps',
while the remainder were all biopsy-positive and aspirate-
negative (authors' unpublished data).

Positive bone marrow biopsy specimens from untreated
patients have not been reported in patients initially clinically
classified as stage I and II A. However, up to 25% of patients
thought to have stage III disease may be reclassified as stage IV
as a result of a positive core biopsy.29,30 Occasionally, marrow
infiltration may be the only evidence of stage IV disease. Bilat-
eral biopsies have been shown to increase the yield of positive
specimens.31

The diagnosis of HD on the basis of marrow infiltration alone
in patients without peripheral lymphadenopathy presents many
difficulties. Neiman et al.26 described a group of 13 patients with
lymphocyte-depleted HD. The patients had fever, pancytopenia,
and abnormal liver function, but there was no striking peripheral
lymphadenopathy. The outcome was consistently fatal, usually
within a short period. In 5 patients the marrow sections were
diagnostic, and in 3 of these were the sole criterion for the
diagnosis.

The occurrence of granulomatous lesions in tissues involved
by HD is well recognized.31 The significance of isolated non-
caseating granulomas in otherwise uninvolved tissues, however,
is less clear. Kadin et al.32 reviewed tissue obtained from 185
patients with HD prior to treatment and noted isolated granulo-
mas in the liver and spleen. Further extensive sectioning of these
tissues failed in many instances to reveal a diagnostic infiltrate of
HD. In some, however, the granulomas were intimately associa-
ted with an HD infiltrate. It was concluded that the discovery of
isolated epithelioid cell granulomas in HD should not influence
the staging criteria.

Another phenomenon occasionally encountered is the pre-
sence of extensive lymphoid aggregates in otherwise normal
biopsy specimens from patients with HD. The significance of
this remains uncertain.

Non-Hodgkin's lymphoma

Proper histopathological classification and clinicopathological
staging are now regularly employed in the non-Hodgkin's lym-
phomas: (i) to define patterns of disease better; (ii) to correlate
these patterns with the clinical course and response to treatment;
and (iii) for selection of the appropriate therapy.

Although aspiration smears or sections of aspirated particles
have been used in the detection of marrow involvement by
lymphoma, several reviews testify to the trephine biopsy as the
best method for detection of marrow lymphoma. By performing bilateral posterior iliac crest biopsies the yield of positive specimens is increased by 10–20%. Marrow biopsy often influences the staging of patients with non-Hodgkin’s lymphoma. It has been shown that 50–65% of patients thought to be in stage I–II may be reclassified as stage IV as a result of a marrow biopsy. A normal full blood count does not exclude infiltration of the marrow.

One of the major problems in the diagnosis of marrow lymphoma is the distinction between benign lymphoid aggregates and lymphoma. Studies of the prevalence of lymphoid follicles in marrow biopsies and clot sections obtained in vitro show a wide variation. Although most biopsy studies (including our unpublished data) report an incidence of 1–9%, much higher figures, up to 47%, are reported when clot sections are used. These benign lymphoid nodules are commoner in older patients and in many cases constitute a normal finding without any known clinical significance. Yet infrequently, however, we have found it difficult to decide whether the marrow was involved by a lymphoproliferative disorder, both in patients with known lymphoma and in those without evidence of lymphoma elsewhere. The following features have been described as suggestive of early lymphomatous infiltration: (i) size of aggregates; (ii) aggregate covering more than 25% of a 40 high-power field is suspect; (iii) cytological atypicality; (iv) multiplicity of aggregates; (v) infiltration into surrounding normal marrow; and (vi) paratrabecular location.

These criteria must be viewed critically, however, as we have seen several marrow specimens with many of the above features in patients in whom subsequent follow-up revealed no evidence of lymphoma. Moreover, nodular lymphoid hyperplasia of the marrow is a well-described entity. Recent reports of this syndrome have emphasized that caution must be exercised before a definitive diagnosis of lymphoma is made. In a study by Ryvlin et al., 10 patients with lymphoid hyperplasia of the marrow were noted. In 5 of these, follow-up was possible. During a 2–4-year period, none of these patients developed signs or symptoms suggestive of a lymphoproliferative disorder. Careful follow-up of patients in whom the isolated finding of lymphoid hyperplasia of the marrow is made is therefore preferable to treating them as cases of early lymphoma.

Diagnosis of the hypoplastic anaemias

Marrow trephine biopsy, preferably from more than one site, is an essential procedure in the diagnosis of hypoplasia. Although this is important diagnostically, no correlation between estimations of cellularity and prognosis has been shown.

Granulomatous disorders (Figs 4 and 5)

Granulomas represent a host inflammatory response which may be the result of a wide variety of stimuli. In an extensive study by Pease of 150 patients with granulomatous lesions in sections of aspirated marrow particles, the commonest underlying disorders were tuberculosis, histoplasmosis, infectious mononucleosis, sarcoidosis, brucellosis, and Hodgkin’s and non-Hodgkin’s lymphoma. Marrow granulomas were also found in a wide variety of other disorders, some of which are not ordinarily associated with granulomatous change. Okun et al. have described the presence of marrow granulomas in a patient with Q fever. Unless a specific organism can be identified by special stains or bacteriological culture, there are no diagnostic features of the granulomatous disorder; although prominent caseous necrosis favours the diagnosis of tuberculosis.

Granulomas may very occasionally be seen in aspirate smears, but the yield is considerably increased by sectioning particles of material. Although no adequately controlled studies are available, biopsy will presumably further increase the yield owing to its ability to reach more deep-seated tissue.

Although marrow aspiration and biopsy are complementary to other investigations, marrow examination and culture may sometimes succeed in establishing the diagnosis of a granulomatous disorder when other tests have failed to do so.

Chronic lymphocytic leukaemia (CLL)

Bone marrow histological patterns and their relationship to prognosis in chronic lymphocytic leukaemia have been evaluated by some authors. Gray et al. studied 115 patients and showed that those with diffuse marrow infiltration had a poorer prognosis than those presenting with a nodular or mixed (nodular and diffuse) pattern. Rozman et al. studied 63 patients with CLL and described 4 different histological patterns: (i) interstitial (lymphoid infiltration without displacement of fat cells); (ii) nodular (abnormal lymphoid nodules without interstitial infiltration); (iii) mixed (combination of the first two patterns); and (iv) diffuse (replacement of both haematopoietic and fat cells by lymphoid infiltration). Statistical analysis of actuarial curves showed a significant difference of survival probability according to the marrow infiltration patterns. In patients with interstitial or nodular patterns life expectancy is significantly less than in those with mixed or diffuse patterns. They also noted a significant degree of correlation between bone marrow infiltration patterns and the various methods of clinical staging.
Miscellaneous

Bone disease. Bone trabeculae can easily be studied in marrow trephine sections and thus provide a simple method for the diagnosis of bone disease, such as may accompany chronic renal failure, intestinal malabsorption, and other disorders.

Lead poisoning. Bone is a primary site of lead storage and core biopsies may therefore be used to estimate stored lead levels. A recent study of skeletal lead concentrations in Americans has documented the presence of plumbism in polluted environments and underlines the value of this investigation in such epidemiological studies.

Contraindications to marrow trephine biopsy

These are all associated with a tendency to increased bleeding. Thrombocytopenic bleeding (Fig. 6) has never been a problem in our experience, provided adequate compression has been applied to the biopsy site on completion of the procedure. In the face of disseminated intravascular coagulation, haemorrhage following a trephine can be catastrophic, and marrow biopsy is therefore contraindicated in the presence of this disorder. In patients with hereditary coagulation factor deficiencies, marrow biopsy should be performed only with adequate cover by the approximate component. For patients on anticoagulant therapy, cessation of therapy is indicated until adequate haemostatic status has been regained.

Fig. 6. Megakaryocytic hyperplasia in an otherwise normal bone marrow specimen from a patient who presented with purpuric bleeding and a platelet count of 5 x 10^9/l. Auto-immune thrombocytopenic purpura was diagnosed.

Complications

The problem of post-biopsy bleeding has been discussed above. The only other complication we have experienced is infection. Our incidence of infection is less than 1%. Infection has occurred only in immunocompromised patients on cytotoxic drugs. We therefore use a strict aseptic technique and wear surgical masks and gloves when biopsying these patients.

Diagnostic pitfalls

Besides some of the diagnostic problems that have already been mentioned, we should like to emphasize a few other problems of interpretation occasionally encountered.

Megaloblastic anaemia

The cellular and apparently blastic appearance of the marrow tissue on sections stained with haematoxylin and eosin may lead to an erroneous diagnosis of leukaemia or lymphoma. The marrow aspirate should obviously lead to the correct diagnosis, but the trephine biopsy may still be misinterpreted by the inexperienced, especially if examined in isolation.

Angio-immunoblastic lymphadenopathy

This unusual hyperimmune disorder of B-lymphocyte origin may involve the marrow. The characteristic morphological features of immunoblastic and plasma cell infiltrates, amorphous acidophilic interstitial material and vascular proliferation may closely resemble HD. The diagnosis is usually made on clinical grounds in conjunction with a lymph node biopsy.

Eosinophilic fibrohistiocytic lesions

These lesions were described by Rywin et al. in 1972. They consist of spindle cells, numerous eosinophils, some plasma cells and occasional mast cells. In Rywin et al.'s series, some of the patients were anaemic and all of them were taking numerous medications. Withdrawal of the drugs led to an improvement in the anaemia and disappearance of the eosinophilic lesions. We have noted a similar lesion in a patient with urticaria pigmentosa.

Post-induction therapy for leukaemia

A trephine biopsy is indicated in the follow-up of patients with acute leukaemia after intensive cytotoxic therapy for the induction of a remission. This applies particularly to acute myeloblastic leukaemia. The trephine biopsy ensures good assessment of: (i) the extent of residual disease, if present; and (ii) the extent of haematopoietic reserve and early signs of regeneration of normal haematopoietic tissue.

Occasionally, diagnostic problems may be encountered in the assessment of biopsy specimens when clusters of blasts are present — is this a sign of residual disease or of regeneration? It is our experience that these sections should be interpreted with caution. Very often these blasts are part of a regenerative process and do not represent residual leukaemia.

We have also noted some unusual regenerative phenomena in classic myeloid leukaemias following induction therapy, e.g. megakaryocytic hyperplasia.

Comment

Needle biopsy of the bone marrow has for some time been the accepted method for obtaining a specimen of bone marrow for examination when aspiration has resulted in a "dry tap". It is only in the last decade, however, that it has become clear that biopsy is superior to aspiration in the detection of Hodgkin's and non-Hodgkin's lymphomas and metastatic malignant tumours. Some authors have advocated the use of sections of aspirated material and have demonstrated their superiority to aspirate smears in the detection of granulomas, metastatic tumours and lymphomas. A comparative study by Dee et al. indicated a distinct superiority of the biopsy specimen when this was compared with the aspirate and sections of aspirated particles in the diagnosis of HD. In non-Hodgkin's lymphomas, biopsy was also the most sensitive diagnostic procedure, particularly with follicular lymphomas, although there were several positive clot sections in the presence of negative biopsies. On the basis of a study performed on 30 cadavers, Rywin concluded that aspirated marrow par-
ticles which were concentrated before sectioning yielded larger areas of marrow for examination than needle biopsy sections. This may be misleading, as 10 ml of marrow was aspirated in each case and this is not always possible in vivo. It is nevertheless clear that the various techniques are complementary. In practice, however, we have found it simpler to obtain and process biopsy specimens as described earlier. Moreover, in contrast to Ryvlin, we are able to obtain equally as good cytological detail in our trephine sections as in the particle sections. It is rare to obtain inadequate specimens, whereas with aspiration inadequate sections for examination may be obtained in as many as 10% of cases. 7

We should like to re-emphasize the importance of proper fixation and preparation of sections. We have frequently received slides which are difficult to interpret owing to poor fixation and thick sections. To obtain good trephine sections is an art which requires some practice and it is therefore preferable to refer patients requiring marrow trephines to centres where biopsies are regularly performed and the specimens examined, rather than submit an inadequate specimen for opinion. This often leads to the patient having to undergo the procedure a second time.

Bone marrow sections should be interpreted by a pathologist or hematologist in conjunction with a thorough examination of the smear preparations. The practice of examining marrow sections in isolation is not recommended and may lead to misdiagnosis.

In summary, therefore, it is our experience that bone marrow biopsy sections with concurrent marrow aspiration smears are complementary, and together allow for a thorough assessment of the bone marrow. The morbidity is minimal and the procedure can be performed on outpatients. The few contraindications to biopsy are those associated with a severe bleeding tendency as a result of depletion of coagulation factors.

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CHOICE OF NEEDLE FOR BONE MARROW TREPHINE BIPSY

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Optimal examination of the bone marrow combines cytology on aspirated material with histopathology, as reflected in the trephine biopsy, where architectural relationships are preserved, with this procedure most usually being carried out using the Westernman-Jensen or Jamshidi needles. Experience with these two has been compared to the recently introduced Islam system and in 50 consecutive studies superior results were obtained with the latter and in no instance was a biopsy of less than 3 cm, devoid of crush artifact, obtained. It is concluded that this latest instrument has advantages over its predecessors and is recommended for routine use.

KEY WORDS: Trephine marrow biopsy, Islam system, Technical aspects.

INTRODUCTION

Simple aspiration, introduced in 1929, is adequate for diagnostic purposes where cells are easily obtained and distinctive cytology exists. In selected cases of nutritional deficiency and the acute or chronic leukaemias, this may be all that is necessary, particularly as it provides a convenient way of obtaining material for immunophenotyping and cytogentic studies.

Conversely, when the marrow cavity is hypercellular and increased reticulin or collagenous fibrosis is present a dry tap results so that trephine biopsy becomes invaluable. Furthermore, there are specific instances where no realistic alternative exists to the latter procedure, exemplified by the need to quantitatively evaluate haematopoiesis in aplastic anaemia, document regeneration following cytotoxic chemotherapy, where questions arise about infiltrative conditions such as the malignant lymphomas and myeloma or in the search for granulomata, as might be found in tuberculosis.

Historically, the first approaches were surgical, but these became outmoded with the introduction of the Vim-Silverman needle and the popular Westernman-Jensen modification. An entirely new method was the Jamshidi tapered device, which has dominated recent practice, although other options exist, including the Gidlund's.
PATIENTS AND TECHNIQUES

Fifty consecutive individuals scheduled for routine diagnostic bone marrow examination, having a wide range of underlying conditions and indications, had the experimental nature of multiple biopsies explained to them and gave informed consent.

Following infiltration of the skin and the tissues overlying the posterior superior iliac spine with local anaesthetic, aspirations were compared using the Turkel and Islam needles. For practical purposes, there was no difference in the quality of material obtained, and in the three patients where the procedure failed it did so using both approaches, and the reason was subsequently shown to be the presence of dense myelofibrosis.

Since it has been suggested that aspiration produces an artifact in subsequent trephine biopsies, this point was examined and our own experience accords with the opposite viewpoint that a one-step combination technique works well and the use of two sites on the iliac crest is therefore not considered necessary.

The technique for biopsy was standardised, and despite a small skin incision having been advocated by some, this was shown to be unnecessary from the first report and in our three decades of experience we would endorse the original opinion. Similarly, whilst the left lateral position was initially advocated and is, indeed, entirely practical, it is easier if the patient is relaxed and prone in a warm procedure area. Interestingly, background music and relaxation exercises are both judged to be significant adjunctive manoeuvres, but their individual benefits have not been objectively documented.

Once the tip of the obturator was firmly anchored in the outer cortex, the needle was advanced, with gentle pressure, on a plane carefully directed towards the ipsilateral anterior superior iliac spine. On penetrating the marrow cavity, generally easily perceptible with even minimum experience, the obturator was withdrawn and a minimum of 3 cm core obtained; anything less than this was designated a failed procedure.

When using the Westerman-Jensen needle, the cutting blades were advanced, with steady pressure, until the desired depth had been reached. At this point, the outer case was rotated until the biopsy was free; this motion was well tolerated if gently carried out and was associated with loss of the least number of cores.

In the case of the Jamshidi needle, it was advanced with a rotating and pronating action through approximately 45 degrees until the required depth was achieved. It was then rotated two or three times in a complete circle in both directions and gradually withdrawn. The modification, said to improve the technique, was not examined in this study. The core was gently delivered through the back of the needle, paying particular attention to the introduction of a correctly sized, solid metal probe supplied for this purpose.

An essentially identical technique was used with the Islam system. Although it has been suggested that the rotation employed for the Jamshidi needle is unnecessary, this causes no discomfort to the patient, favours optimum penetration, achieves a smooth cut to the edges of the biopsy and universal retention of the core.
RESULTS

The Westerman-Jensen Needle

Based on our previous experience\textsuperscript{2}, we have largely overcome technical failure, although in this series of 50 patients adequate material was not obtained in four individuals. In two, this was because the bones were virtually impenetrable due to osteosclerosis, and in two, unacceptably small traumatised cores were obtained in the face of dense myelofibrosis. Despite care in carrying out the procedure, the cutting blades were widely splayed in all four of these patients and in two were effectively destroyed.

Maintenance of this instrument is relatively time-consuming and requires that the cutting surfaces be gently rubbed with the finest possible water paper to ensure a sharp edge after every two or three procedures. Any distortion of the delicate blades renders biopsies difficult and leads to an immediate increase in crush artifact. However, with meticulous attention to detail and as experience accumulates, these needles have a long life and our record with one set now exceeds 300 biopsies.

The Jamshidi Needle

This resulted in the core being unobtainable on the first try in seven patients, and in five this failed on two further attempts. In contrast to the Westerman-Jensen needle, the biopsies were recovered in patients with sclerosis and fibrosis, but were lost in three who had profound osteoporosis and in an additional two where the bone was soft and gelatinous due to myelomatous infiltration.

The maintenance of the angled cutting edge is crucial. However, inevitably, and even with precision engineering, there is loss of the taper and gradual gaping of the mouth; from this point, biopsies are readily lost, particularly with soft marrow. In our experience, the life of the Jamshidi needle, with optimal care, is somewhere between 100 and 150 procedures, with minimal resharpening being needed about every 10 to 15 biopsies.

The Islam Needle

In all 50 patients, adequate biopsies were obtained. A learning curve of only two or three procedures was needed, and even these were easy to carry out and suitable samples obtained. Of particular note was the fact that biopsies were readily straightforward with dense myelofibrosis at one end of the spectrum and tumour replacement with myeloma and serous atrophy or gelatinous necrosis at the other.

To date, we have little experience in maintaining the serrated cutting edge, with the current recommendation being to return these to the factory for sharpening. Having deliberately used a single needle for all these procedures, absolutely no loss of quality or increase in crush artifact has been noted. It would appear as though somewhere between 100 and 200 biopsies should be achievable before resharpening is necessary, and it may well be that in many areas this can be carried out locally and at relatively low cost.
DISCUSSION

Routine diagnostic aspiration from the posterior iliac crest or the sternum was judged to be no different using the Turkel or the particular Islam needle designed for this purpose. Some concern does, however, exist about the use of the latter for the sternum, where the guard is held in position with a simple screw as opposed to the more secure threaded system available on some of the alternatives. This argument does not apply to aspiration from the posterior iliac crest, but here neither the volume nor the quality obtained differed from instruments used in the comparison.

With regard to biopsies, the Westerman-Jensen needle, although well tried and compared to the Jamshidi systems, is limited by the pliability of the blades, and in anything other than marrow of near normal texture these tend to become distorted and on occasions impossible to restraighten. Furthermore, crush artifact is relatively frequently encountered, even when the needles are maintained in excellent condition by sharpening them regularly with fine grain water paper. Of the three needles tested, this is the easiest one to introduce and engages the cortex most readily, so that sliding or shifting in the first part of the procedure is not a problem. Despite shortcomings, it is excellent for soft or gelatinous medullary contents, but has a significant failure rate in the presence of fibrosis or dense infiltrative lesions.

The Jamshidi needle, which has been popular for some years now, bears many similarities to the Islam system. It does, however, have two limitations. The first of these is that the maintenance of its cutting edges is difficult, and even with polishing or minimal resharpening the taper is gradually lost, so that the obturator fits less well and cores are increasingly left behind in the patient as the mouth of the needle widens.

However when new or little used they are easy to employ and produce good biopsies in most instances, although retention of very soft or necrotic cores is a definite problem, requiring repeat procedures.

The Islam system has been uniformly more versatile and has the highest success rate of the three. No cores were lost, crush artifact was, for the most part, nonexistent, and neither soft nor hard medullary contents posed the problems seen with the alternatives. The extent to which maintenance of the serrated cutting edge will create difficulties is presently unknown. In practical terms, the recommendation by the suppliers that these be returned to the factory for precision engineering after approximately 100 procedures remains an issue for clarification.

CONCLUSION

Aspiration with the Islam needles for cytormorphology is equivalent to standard and established techniques; its additional cost does not appear justifiable in these circumstances. In contrast, the trephine biopsy system was more flexible and gave consistently superior results in this study when compared to the older Westerman-Jensen and Jamshidi counterparts, with crush artifact and loss of cores not being encountered.

The technique for the use of these needles proved easy to impart to residents in training. Based on this small but direct comparative study, this system can be recommended as that of choice for routine diagnostic laboratories. It is safe and efficient, with skills not being lost, even when procedures are infrequently carried out. In services performing large numbers of biopsies, as with the Westerman-Jensen and Jamshidi needles, it is
mandatory to maintain a clean and sharp cutting edge. We are able to comment on whether this could be achieved by local engineers or would require recycling to the factory, probably after 100 to 200 procedures.

Acknowledgements

We thank Jessica Gerretsen for bibliographic assistance and Jackie Davies for help with preparation of the manuscript and its typing.

References


EFFECT OF ANTHRACYCLINES ON MYOCARDIAL METABOLISM AND CALCIUM TRANSPORT AND A COMPARISON OF TWO ANALOGUES IN EQUIMOLAR CONCENTRATIONS

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*MRC-UCT Ischaemic Heart Disease Laboratory and the
**University of Cape Town Leukaemia Centre and the Department of Medicine and Haematology, University of Cape Town and Groote Schuur Hospital, South Africa

Summary

10-20 μmol/L carminomycin produced more severe acute heart failure than equimolar concentrations of daunomycin (P<0.04) in the isolated perfused working rat heart. Coenzyme Q10 (Q10), which is an obligatory coenzyme of oxidative phosphorylation, was unable to restore control cardiac output although Q10 restored (P<0.001) ATP depletion which was associated with anthracycline administration. We therefore concluded that heart failure was not due to ATP depletion. Reversal of heart failure (P<0.0001) by 2.5 mM to 5.0 mM change in the perfusate calcium concentration suggested that anthracycline acute cardiotoxicity might be on the basis of calcium channel antagonism. Lack of lactate dehydrogenase release into the medium suggested that myocardial cell membrane damage did not occur.

Cardiotoxicity limits the use of anthracyclines, which are potent antimitotic agents, used to treat solid tumours and leukaemias (2,3,13). Impaired myocardial metabolism (14,17) or altered calcium channel activity (1) has been proposed to explain anthracycline cardiotoxicity. In addition to comparing equimolar concentrations of a new analogue, carminomycin to daunomycin on the isolated perfused working rat heart model, we studied the possible role of calcium channel activity by manipulating the perfusate calcium concentration.

Myocardial metabolism was evaluated directly by measuring tissue adenine triphosphate (ATP) of the hearts and indirectly by administration of a mitochondrial extract Q10. Release of lactate dehydrogenase (LDH) into the medium was taken as a non-specific index of cell membrane damage (5,6,7).

Materials and Methods

Hearts, excised from Long-Evans male rats and rapidly arrested in Krebs-Henseleit buffer at 4°C, were preperfused via the aorta by the Langendorff method at a hydrostatic pressure of 65 cm H2O while the pulmonary veins were cannulated. After 15 minutes of Langendorff perfusion, hearts were perfused by the left atrial method (10) for 75 minutes at a filling pressure of 10 cm H2O, working against a hydrostatic pressure of 100 cm. The perfusate was a Krebs-Henseleit (9) bicarbonate medium equilibrated with 5% CO2, 5% O2, with 11 mM glucose as external substrate, with an ionic composition of NaCl 128.5 mM, KCl 4.7 mM, CaCl2 2.5 mM, K2HPO4 1.19 mM, MgSO4 1.19 mM and NaHCO3 25 mM. 5.0 mM CaCl2 perfusate was obtained by doubling the CaCl2 added to the solution. Release of LDH from the heart was measured as the arteriovenous difference.

Solutions prepared from the mannitol-stabilized powder of daunomycin HCl and carminomycin HCl, were added to the atrial perfusion at 20 min. of the working heart. 10 μg/kg of vehicle-free Q10 was administered via the tail vein at 2 hours and at half an hour before sacrifice, in equally divided doses.

At the end of experiments, hearts were freeze-clamped by aluminium tongs (16),
cooled to the temperature of liquid nitrogen and analyzed for ATP and phosphocreatine (PCR) (11) expressed in terms of fresh weight.

Results were expressed as means ± standard error of the mean, with P values calculated from Student's t test and values greater than 0.05 were considered not significant.

Results

Table 1 shows results (means ± SEM) at 55 minutes after addition of anthracyclines:

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>17.5μmol/l</th>
<th>Daunomycin</th>
<th>Carminomycin</th>
<th>Q10 + Carminomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>58 ± 1.0</td>
<td>32 ± 2.4</td>
<td>44 ± 1.6</td>
<td>25 ± 2.1</td>
<td>25 ± 2.1</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>μl/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>3.7 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>3.3 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>μmol/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>5.1 ± 0.8</td>
<td>3.2 ± 0.2</td>
<td>4.0 ± 0.4</td>
<td>4.6 ± 0.4</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>μmol/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LDH</td>
<td>8.0 ± 0.8</td>
<td>7.0 ± 1.0</td>
<td>5.0 ± 0.3</td>
<td>5.0 ± 1.3</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>mlU/g/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

N = 8 for all groups
a P<0.04 carminomycin vs equimolar daunomycin
b P<0.0003 vs control
c P<0.003 vs control
d P<0.001 vs 17.5 μmol/l carminomycin

In Table 1 10-20 μmol/l of daunomycin and carminomycin produced time and concentration dependent mechanical failure as measured by cardiac output. In equimolar concentrations carminomycin was more cardiotoxic than daunomycin (P <0.04). Q10 did not influence mechanical function of hearts treated with 17.5 μmol/l carminomycin.

17.5 μmol/l of daunomycin and carminomycin significantly (P <0.003) reduced ATP values from control values. ATP, depleted by 17.5 μmol/l carminomycin was significantly (P <0.001) restored in hearts that were pretreated with Q10.

In figure 1 the reduction of cardiac output produced by carminomycin was much less (P <0.0001) when the perfusate calcium ion concentration was doubled. The protection lasted throughout the experiment. Daunomycin was not tested at the higher calcium ion concentration.

Data in figure 1 has been included in a detailed manuscript prepared for Cancer Research, Saman, Opie, Jacobs (1982).
FIGURE 1: 5.0 mM Ca^{2+} reversed mechanical failure produced by 17.5 μmol/l carminomycin (CMH).

\[
\text{--- 17.5 μmol/l CMH } + 5.0 \text{ mM Ca}^{2+}.
\]

\[
\text{----- 17.5 μmol/l CMH } + 2.5 \text{ mM Ca}^{2+}.
\]

Results are means ± SEM (n).

▲ P < 0.0001 vs control. * P < 0.0001 vs 2.5 mM Ca^{2+} + 17.5 μmol/l CMH.
The reduction of cardiac output when 17.5 μmol/l CMH was added, was much less for 5.0 mM Ca^{2+} concentration.

Discussion

The proposal that anthracyclines produce acute heart failure by inhibiting the synthesis of high energy phosphates (12,14) is unlikely in our preparation because firstly, phosphocreatine was preserved and, secondly, Q10 was unable to reverse acute heart failure by restoring ATP depleted by the anthracyclines. In another study of the isolated rat heart, high energy phosphates were preserved even though heart failure occurred (4).

Our study also does not support the possibility that anthracyclines produced heart failure by chelating extracellular calcium ions (8). Calculation reveals that in our experiments each anthracyclines molecule would have to bind approximately 70 calcium ions to reduce the perfusing medium (intracellular) calcium ion concentration by half.

It is more likely that anthracyclines produced acute heart failure by blocking
slow calcium channels. Support for this hypothesis stems from: firstly, our finding that increase in the calcium ion concentration of the perfusing medium protected against anthracyclines heart failure; secondly, recent evidence that high doses of anthracyclines blocked the slow response action potential (1); and thirdly, evidence that pretreatment of rats with digoxin protected the isolated perfused heart from daunomycin cardiotoxicity (4).

Heart failure in our study was unaccompanied by myocardial enzyme release suggesting that myocardial membrane damage did not occur.

In equimolar concentrations, the new analogue, carminomycin, proved to be more cardiotoxic than daunomycin. However, if it is confirmed that carminomycin has a better antimitotic efficacy at a lower concentration, then the true chemotherapeutic dose might be less.

In conclusion we propose that either calcium ion infusion or digoxin pretreatment (4) might be of value in preventing acute anthracycline cardiotoxicity.

Acknowledgements

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References

Mechanism of Acute Anthracycline Cardiotoxicity in Isolated Rat Hearts: Carminomycin versus Daunomycin

Selva Saman, Peter Jacobs, and Lionel H. Opie

ABSTRACT

Cardiotoxicity limits the use of anthracyclines which are potent antitumor agents. In the isolated rat heart, we investigated the mechanism of acute anthracycline cardiotoxicity and compared a new anthracycline, carminomycin, with daunomycin which is in established use. Daunomycin 1.75 × 10^{-9} M produced a fall in cardiac output (36 ± 2 versus 58 ± 1 ml/min; p < 0.01), left ventricular power production (9 ± 0.7 versus 16 ± 0.3 mJ/sec/g; p < 0.01), and efficiency of heart work (3.3 ± 0.2 versus 6.3 ± 0.2 mJ/sec/ml O_2; p < 0.01; mean ± S.E. 40 min after daunomycin). Carminomycin (1.75 × 10^{-8} M) produced a greater fall in cardiac output than equimolar daunomycin (26 ± 2 versus 36 ± 2 ml/min; p < 0.01). Daunomycin did not reduce coronary flow rate, heart rate, or oxygen consumption. From the preceding data, we inferred that, since afterload and preload were constant in this model, heart failure was due to a depressed inotropic state. Procedures that increased cytosolic calcium relieved heart failure namely, pretreatment with digoxin (62.4 μg), isoproterenol (10^{-8} M), and increased perfusate Ca^{2+} (5 mm versus 2.5 mm) all prevented carminomycin-induced fall in cardiac output (41 ± 1, 47 ± 5, and 52 ± 1, respectively, versus 26 ± 2 ml/min; p < 0.01). Acute anthracycline contractile failure was also associated with a fall in high-energy phosphate compounds which could also have contributed to the decreased inotropic state. We conclude that carminomycin is more cardiotoxic than daunomycin in equimolar concentrations and that a lowered cytosolic calcium and decreased energy stores might cause the contractile failure. The cytosolic calcium and high-energy phosphate compounds were lowered by separate mechanisms.

INTRODUCTION

Anthracyclines are potent antitumor agents (29). Their use may be limited by cardiotoxicity of which 2 types have been identified. (a) Acute clinical cardiotoxicity with a fall of ejection fraction can be found within 4 to 24 hr of administration of anthracyclines in patients (3, 26). (b) With cumulative doses of anthracycline, a chronic toxic cardiomyopathy may occur (17). There may be a common mechanism for the acute and chronic cardiotoxicity because the myocardial lesions were similar in one study (31). This paper investigates the mechanism of acute contractile failure in an isolated heart model. We also compare daunomycin with equimolar concentrations of carminomycin (6), a new analogue reported to be less cardiotoxic (7).

Previously contractile failure in blood perfused hearts has been explained by a decreased coronary flow rate (19). However, the finding of ATP depletion in cultured cells exposed to Adriamycin (25) argues against a simple effect on coronary flow and suggests a direct toxic effect on energy production (10). Another hypothesis is that anthracyclines might cause myocardial cell membrane damage and thereby cause enzyme release (9, 22). Another possible hypothesis is that there is a decreased availability of cytosolic calcium, because anthracyclines may block membrane Na^{+}/Ca^{2+} exchange (5) or antagonize calcium-dependent slow-response action potentials (1). Cardiac glycosides are reported to prevent acute contractile failure caused by anthracyclines (4, 33), although they neither compete with anthracyclines for membrane binding sites (27) nor prevent myocardial uptake of anthracyclines (2). Because cardiac glycosides are thought to increase cytosolic calcium (14, 20) indirectly, these observations support the proposed role of a decreased cytosolic calcium in acute contractile failure.

We explored the mechanism of acute anthracycline cardiotoxicity by the following procedures: (a) the role of coronary flow was studied by relating coronary flow rates to myocardial mechanical performance; (b) the possible role of myocardial energy metabolism was evaluated by measuring the tissue content of high-energy phosphates and of cyclic AMP; (c) the possible toxic effect of anthracycline on mitochondria was indirectly studied by observing the effects of coenzyme Q_{10}, an agent thought to stabilize the mitochondrial membrane (18); (d) release of lactate dehydrogenase into the perfusing medium was used as a non-specific index of cell membrane damage; and (e) the possible role of calcium ions was studied by altering the perfusate calcium concentration or by administration of isoproterenol or digoxin.

MATERIALS AND METHODS

Isolated Working Heart. Hearts (exsised from male Long-Evans rats; weight, 250 to 300 g) were rapidly arrested in Krebs-Henseleit buffer at 4 °C. The hearts were preperfused via the aorta by the Langendorff method at a hydrostatic pressure of 65 cm H_{2}O for 15 min and were thereafter perfused by the left atrium (21) for a further 75 min at a filling pressure of 10 cm H_{2}O, working against a hydrostatic pressure of 100 cm; 100 ml of perfusing medium were recirculated. The perfusate was a Krebs-Henseleit (16) bicarbonate buffer equilibrated with 95% O_{2} and 5% CO_{2}, with 11 mmol glucose as external substrate, and with an ionic composition of NaCl, 115.5 mm; KCl, 4.7 mm; CaCl_{2}, 2.50 mm; KH_{2}PO_{4}, 1.19 mm; MgSO_{4}, 1.19 mm; and NaHCO_{3}, 25 mm. CaCl_{2} (5.0 mm) was obtained by doubling the CaCl_{2} added to the solution.

Aortic pressure was measured by a Statham P23DB pressure transducer and monitored on a Device M2 direct writer, Welwyn Garden City, Hertfordshire, United Kingdom. Heart work (W) or power production (13) was measured as the sum of pressure and kinetic power by:

\[ \text{Pressure power} = W_p = 0.002222 \times P \times CO \]

\[ \text{Kinetic power} = W_k = \frac{1}{432 \times 10^5} \times \frac{a(CO)^3}{A^2} \times \left( \frac{I}{T_0} \right)^2 \]

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where $P_s$ is pressure power, $P_o$ is peak systolic pressure, $CO$ is cardiac output, $W_k$ is kinetic power, $d$ is density of perfusate; $A$ is internal cross-sectional area of aortic cannula, $T$ is cycle time, and $Te$ is ejection time. Units are milliwatts (mW/sec) for kinetic power, mm Hg for pressure power, ml/min for cardiac output, g/cm for density, and sq cm for area. The perfusate density was taken as 1 g/cm³.

The efficiency of mechanical work was the total power production (pressure plus kinetic) divided by the oxygen uptake.

Release of lactate dehydrogenase from the heart was measured as the arteriovenous difference (AV) at 5, 15, 30, and 60 min of left atrial perfusion.

The required concentrations of daunomycin and carminomycin (1 × 10⁻⁴ M and 1.75 × 10⁻⁵ M) were obtained in the perfusate by addition of a solution to the arterial perfusion at 20 min of working heart. Solutions were prepared from the mannitol-stabilized powder of daunomycin HCl and carminomycin HCl. In one series, rats were pretreated with coenzyms 

Drugs. Daunomycin and carminomycin were obtained from Maybaker, Port Elizabeth, South Africa, and Bristol-Myers Co., New York, NY, respectively.

Statistical Analysis. Results were expressed as mean ± S.E. Statistical analysis was by one-way analysis of variance or 2-way analysis of variance as indicated; p values greater than 0.05 were considered not significant.

RESULTS

Control Working Heart. In control hearts, cardiac output and coronary flow rates were stable during the 75-min perfusion period that hearts were made to work (Chart 1). At 60 min, left ventricular power production and efficiency were the same as 15-min values (Chart 2). Heart rate was 244 ± 5 at 5 min but stabilized at between 220 and 230/min at 15 min. Left ventricular stroke volume was 0.24 ± 0.01 ml at 5 min and stabilized at 0.26 ± 0.01 ml at 15 min. Peak aortic systolic pressure was not changed for the duration that hearts worked. Oxygen consumption was 185 ± 4, 164 ± 6, and 153 ± 4 units/g/min at 15, 30, and 60 min. Phosphocreatine, ATP, and cyclic AMP values were 5.1 ± 0.8 μmol/g, 4.7 ± 0.2 μmol/g, and 0.40 ± 0.02 nmol/g, respectively (Table 1), at the end of the perfusion period (75 min).

Effect of Daunomycin and Carminomycin. Daunomycin and carminomycin, which were added 20 min after the onset of heart work, produced mechanical heart failure which was concentration related (Chart 1). Fifty-five minutes after daunomycin and carminomycin (1.75 × 10⁻⁵ M), cardiac output was reduced by 44 and 56% respectively. Left ventricular stroke volume was reduced by 42 and 56%, and peak aortic systolic pressure was reduced by 12 and 16%, respectively (Table 1). Heart rate did not fall, so that the decreased cardiac output was caused by a fall in the stroke volume. At 2 equimolar concentrations, carminomycin produced greater fall in cardiac output (p < 0.01) than daunomycin. Coronary flow rates were not reduced. Left ventricular power production and efficiency of work were reduced by addition of Anthracycline
Table 1
Effect of anthracyclines (55 min) and added Q<sub>0</sub> on myocardial energy metabolism and mechanical function

<table>
<thead>
<tr>
<th></th>
<th>ATP (µmol/g, wet wt)</th>
<th>Phosphocreatine (µmol/g, wet wt)</th>
<th>Cyclic AMP (nmol/g, wet wt)</th>
<th>Peak systolic pressure (% of predrug value)</th>
<th>Stroke volume (ml)</th>
<th>Heart rate (beats/min)</th>
<th>Cardiac output (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1 ± 0.8</td>
<td>0.40 ± 0.04</td>
<td>101 ± 1</td>
<td>0.26 ± 0.01</td>
<td>223 ± 5</td>
<td>57.1 ± 0.8</td>
</tr>
<tr>
<td>Daunomycin (n = 8–16)</td>
<td>5.2 ± 0.3</td>
<td>5.5 ± 0.4</td>
<td>0.40 ± 0.02</td>
<td>102 ± 2</td>
<td>0.24 ± 0.01</td>
<td>228 ± 10</td>
<td>52.9 ± 1.3</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt; × 1 µ</td>
<td>3.1 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.2</td>
<td>0.35 ± 0.02</td>
<td>88 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.15 ± 0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>220 ± 9</td>
<td>39.0 ± 2.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Camomycin (n = 8–16)</td>
<td>3.3 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.7 ± 0.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.36 ± 0.02</td>
<td>97 ± 2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.20 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>219 ± 3</td>
<td>44.3 ± 1.6&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>10&lt;sup&gt;-5&lt;/sup&gt; × 1 µ</td>
<td>2.9 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>4.8 ± 0.4</td>
<td>0.36 ± 0.02</td>
<td>96 ± 2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.14 ± 0.01&lt;sup&gt;f&lt;/sup&gt;</td>
<td>136 ± 11</td>
<td>26.7 ± 2.0&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Coenzyme Q&lt;sub&gt;0&lt;/sub&gt; +</td>
<td>4.2 ± 0.1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.6 ± 0.2</td>
<td>0.12 ± 0.01&lt;sup&gt;h&lt;/sup&gt;</td>
<td>214 ± 12</td>
<td>24.7 ± 2.3&lt;sup&gt;h&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a</sup> Mean ± S.E.
<sup>b</sup> p < 0.001 versus control. Statistical analyses by one-way analysis of variance.
<sup>c</sup> p < 0.01 versus control.
<sup>d</sup> p < 0.05 versus control.
<sup>e</sup> p < 0.001 versus camomycin 10<sup>-5</sup> × 1.75 µ.
<sup>f</sup> Absence of data.

Table 2
Oxygen consumption and lactate dehydrogenase release related to cardiac output 40 min after anthracycline was added

<table>
<thead>
<tr>
<th></th>
<th>O&lt;sub&gt;2&lt;/sub&gt; consumption (µmol/min)</th>
<th>Lactate dehydrogenase release (milliunits/mg/min)</th>
<th>Cardiac output (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>153 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 1.3</td>
<td>57.6 ± 1.0</td>
</tr>
<tr>
<td>Daunomycin (10&lt;sup&gt;-4&lt;/sup&gt; × 1.75 µ)</td>
<td>168 ± 8</td>
<td>7.6 ± 1.3</td>
<td>36.2 ± 1.8</td>
</tr>
<tr>
<td>Camomycin (10&lt;sup&gt;-5&lt;/sup&gt; × 1.75 µ)</td>
<td>147 ± 6</td>
<td>5.0 ± 1.2</td>
<td>25.9 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 13 to 16.
<sup>b</sup> Mean ± S.E.
<sup>c</sup> p < 0.001 versus control, statistical analysis by one-way analysis of variance.

Chart 3. Protection by higher perfusate Ca<sup>2+</sup> (5 mm versus 2.5 mm) (n = 8), isoproterenol (n = 5), and digoxin (n = 6) against the fall of cardiac output produced by camomycin (2) (n = 16). Calcium concentration other than as indicated was 2.5 mm. Control, n = 14. Coenzyme Q<sub>0</sub> series, n = 7. * p < 0.01 versus camomycin 1.75 × 10<sup>-6</sup> µ alone.

48 and 42%, respectively, after added daunomycin (40 min; Chart 2). Similarly, camomycin reduced power and efficiency by 64 and 61%, respectively. However, the oxygen consumption was not altered by either daunomycin or camomycin (Table 2). Fifty-five min after added daunomycin or camomycin, the fall in ATP was 34 or 38%, and the fall in phosphocreatine was 37 or 10% (not significant), respectively.

Effect of Interventions. The following interventions were tested: (a) a higher perfusate calcium ion concentration (5 mm versus 2.5 mm); (b) addition of isoproterenol to the perfusate; and (c) pretreatment of rats with either digoxin or coenzyme Q<sub>10</sub>. The aim was to prevent the fall in cardiac output caused by camomycin (1.75 × 10<sup>-6</sup> µ, p < 0.01 versus camomycin 1.75 × 10<sup>-6</sup> µ alone).

Chart 4. Effect of 5 mm perfusate Ca<sup>2+</sup> (n = 8) on fall of left ventricular power production (Prod.) (top) and efficiency of heart work (bottom) produced by camomycin 1.75 × 10<sup>-3</sup> µ (n = 14). Control (n = 14) perfusate Ca<sup>2+</sup> was 2.5 mm, * p < 0.01 versus camomycin 1.75 × 10<sup>-6</sup> µ alone.
whereas digoxin pretreatment gave 74% of the control. The high calcium treatment also partially prevented the fall in left ventricular power production and efficiency of work caused by cami-
nomycin (Chart 4).

Release of Lactate Dehydrogenase. Low levels of enzyme release were found at 60 to 75 min after the onset of atrial perfusion; higher values in the first 15 min were probably related to the trauma of excision and mounting of the hearts (8). Neither daunomycin nor camininomycin increased enzyme release over the whole time course of perfusion; results at 75 min are shown in Table 1.

DISCUSSION

An early hypothesis for the acute myocardial contractile failure caused by anthracyclines was that of coronary vasoconstriction. Mhatre et al. (19) proposed that an anthracycline metabolite, which required blood for its production, was responsible for contractile failure. Yet, when their isolated hearts were perfused with a blood-free solution, the coronary perfusion pressure did not change. In our hearts perfused by an artificial medium, there was contractile failure even though the coronary flow did not fall (Chart 1). Since the preload (left atrial pressure) and afterload (height of perfusion column) were unchanged, and heart rate did not fall (Table 1), the acute heart failure could be ascribed to a reduced inotropic state rather than to any coronary vasocstriction.

A disturbance of cellular energy stores could play a role, as suggested by recent studies with nuclear magnetic resonance in isolated rabbit hearts (12). Anthracyclines might impair the production of high-energy components by acting on the mitochondria (24). In our hearts, the contractile failure produced by treatment with anthracyclines was associated with a fall in ATP, but it is unlikely that fall in ATP was the only factor causing the contractile failure, since coenzyme Q_10 restored the high-energy phosphate compounds toward normal but did not improve mechanical function (Table 1).

Anthracyclines may depress the inotropic state by modulating the cytosolic calcium concentration. This hypothesis was supported by our finding that procedures which increased cytosolic calcium relieved heart failure, namely, higher perfusate calcium, isoproterenol, or digoxin. A higher perfusate calcium could increase the inward flux of calcium and thereby elevate the cytosolic calcium ion concentration. Isoproterenol might do this by enhancing calcium entry by the slow inward current (28), whereas digoxin might achieve the same effect by inhibiting the sarcoplasmic Na^+K^-ATPase (2, 11, 32). The negative inotropic effect of acute anthracycline administration has also been reported in in vitro preparations studying contractile force (1, 15, 19, 32). The mechanism could be that anthracyclines either sequester calcium at intracellular sites (24) or interfere with the transmembrane flux of calcium ions (5). It is unlikely that anthra-
cyclines altered calcium flux by modulating cyclic AMP, because the myocardial levels of the latter were unchanged.

We conclude that, in our model, anthracyclines lowered cyto-
solic calcium ion concentration and impaired the production of high-energy phosphates by separate mechanisms. It must be emphasized that our conclusions, based on the acute effects of anthracyclines on an in vitro heart preparation, may not neces-
sarily be relevant to the situation in patients receiving anthracy-
clines.

ACKNOWLEDGMENTS

We thank Bristol Myers for the donation of caminomycin. Maybaker for da-
nomycin, Catherine Hoog for technical assistance, and Susan Abraham for the illustrations.

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NEW PLATELET MEASUREMENTS

To the Editor: The critical reassessment of old, well-established haematological methods has, inevitably, followed the introduction of dynamic measurements into the study of blood and its diseases. An important benefit of reviewing old concepts in the light of new data is the opportunity of assigning a more meaningful interpretation to the same observations, so that a clearer understanding of the disturbed pathophysiology emerges than may be appreciated from casual or superficial examination.

Perhaps the classic example of this approach is the way in which the ordinary reticulocyte count may be more meaningfully expressed. Thus, by taking into consideration the degree of anaemia and prolonged maturation of these cells in the circulation, a reticulocyte index can be derived, and this correlates well with red cell production. The revised measurement reliably reflects effective erythropoiesis, making possible a distinction between hypo-proliferative anaemia and that due to accelerated loss of mature cells from the circulation, and this without recourse to the more complex and less readily available kinetic measurements.

An analogous situation in which a simple laboratory test can yield a wealth of additional information once its full potential is realized, holds true for the platelet. Thus, functional and morphological heterogeneity have been clearly demonstrated, with young platelets at one end of the spectrum being indistinguishable by their larger size and greater volume. The latter, now known as megathrombocytes, account for roughly 10% of the number normally present on the stained smear: when this number increases, a correlation with stimulated thrombopoiesis and accelerated platelet production is demonstrable.

Since the megathrombocytes have a characteristic appearance on the stained blood film, a routine laboratory technique is readily available for the assessment of platelet production without necessarily subjecting the patients to the inconvenience of full isotopic thrombokinetic study. A further simple refinement, also readily available, is evaluation of platelet volume with electronic sizing equipment, and this will add a confirmatory dimension to simple examination of the stained smear.

The clearer appreciation of the benefits that may be derived from critical examination of platelet morphology and size is reflected in the examination of the patient with thrombocytopenia. In this situation it should be possible to divide the individuals into two broad groups, one on the basis of a production defect where these platelet parameters will be normal, and a second where accelerated peripheral removal with an intact marrow will be identified by an increased number of large platelets.

We have recently examined a series of such individuals, and our results are essentially in agreement with those described by Garg, Lackner and Karpatic, who demonstrated significant increase in megathrombocytes in patients with systemic lupus erythematosus (68%, of 41 patients), chronic auto-immune thrombocytopenic purpura in apparent remission (47% of 14 patients), disseminated intravascular coagulation (52% of 10 patients), individuals with rheumatic heart disease (48% of 19 patients) and those with prothrombotic heart valves and rheumatic heart disease (20% of 25 patients). The significance of these findings serves to emphasise the earlier report from Harker and Schlichter, who demonstrated a much shorter bleeding time in thrombocytopenic patients when this occurred on the basis of accelerated turnover, than when low platelet counts reflected impaired production. The logical extension of this latter observation is the prediction that clinical deterioration characterised by increased numbers of megathrombocytes, which could easily be identified by examination of the blood film, would indicate a predisposition to a thrombotic state.

While it is logical to incorporate the increased number of circulating megathrombocytes in clinical situations associated with clotting abnormalities, and to seek such at-risk patients with screening procedures, a degree of caution should be injected into an unqualified approach, since it is not yet proved that such a correlation is absolute.

In an attempt to define those situations where the more sophisticated tests are needed, prospective studies are in progress to relate platelet size and volume to accelerated turnover in patients who have had prosthetic cardiac valves inserted. In addition to further clarifying this relationship, such observations must be applied to examining the hypothesis that drugs known to interfere with platelet function will return the disturbed platelet homeostasis towards normal and, therefore, provide a rational basis for the selection and use of different anticoagulant regimens in this group of patients.

Obviously, then, the time has come when routine haematology laboratories should be extracting considerably more information from the platelet count and morphology than may have been general practice to date, and this would be particularly true in the elucidation of bleeding and clotting problems. Equally clearly, such observations need to be coupled with more refined measurements of thrombopoiesis in order that the limitations of the former, in characterizing dynamic pathophysiological processes, may be more precisely appreciated.

Finally, in general terms, the need clearly exists constantly to re-examine tests in routine use with more sophisticated measurements so that, with the reticuloocyte count and platelet morphology, optimal interpretation of otherwise static measurements can be made available by the laboratory to the clinical staff.

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DRUGS IN OBESITY

ERRATUM

To the Editor: We refer to our letter on drugs in obesity, which was published in the SAMJ on 10 May 1975. We should like to point out a small but important error. The fourth paragraph should start: 'Of the 32 subjects who completed the trial, I complained of insomnia and headache 1 of nausea and 1 of agitation — side-effects which did not necessitate discontinuation of the drug'.

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A. L. Vinik

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assessed. However, these preliminary results suggest that this type of system may be a more valid model than that provided by experimental animal tumours for the laboratory study of clinical response to chemotherapy.

Acknowledgment: I am grateful to Dr Myrtle Gordon at the Institute of Cancer Research, Sutton, for allowing me to use her data in Figure 3.

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A practical method for ensuring long-term venous access

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Reliable venous access is of critical importance in the management of the chronically ill and debilitated patient. It is also indispensable during the period when cytotoxic chemotherapy is infused for induction of remission in patients with acute leukaemia and following bone marrow transplantation, when the patients may depend upon intravenous therapy continuously for 6 weeks or more. In these circumstances, consideration must be given to the risks of introducing infection that follow repeated venepunctures in immunocompromised patients, while movement for nursing procedures and physiotherapy inevitably disturb the safety of a peripherally-located venous line.

In an attempt to resolve these problems a technique was developed based on earlier studies in a rabbit model (Jacobs 1973, Jacobs & Adriaenssens 1970) where an entirely subcutaneous plastic system had been implanted; the reservoir remained available for intermittent sampling and provided intravenous access over a 3-month period. The same method is equally applicable to man, but we have recently modified this because, following irradiation and bone marrow transplantation, continuous as opposed to intermittent venous access is mandatory.

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On the basis of our first 30 procedures, we report a simple technique that has been found helpful in this regard since it leaves the arms free, does not expose the patient to the risks of repeated venepunctures, and is suitable both for intravenous infusion and blood sampling over a long period of time. In contrast to percutaneous subclavian catheterization this approach has been totally free of any complications.

**Technique**
The venous line can be placed under either general or local anaesthetic. A 10-gauge silastic paediatric nasogastric tube with a rounded solid tip and two side holes has been found to be most suitable.

A 1 cm incision is made diagonally across the deltopectoral groove at the junction of its upper and middle third. To locate the groove the flat hand is placed on the anterior chest wall and moved laterally over the pectoralis major, when the tips of the fingers will palpate the medial fibres of the deltoid as a ridge. This manoeuvre identifies the position of the cephalic vein (Figure 1). It should be remembered that this groove harbours not only the cephalic vein but also the vessels from the acromiothoracic trunk which lies deep to the vein in that situation (Figure 2). The catheter is introduced subcutaneously through a small stab wound 3 cm lateral to the skin incision and enters the subclavian vein via a small incision in its cephalic tributary (Figure 3). In rare circumstances the basilic vein may have to be used instead of the cephalic, but the principle remains the same. The catheter is directed to lie with its tip 1 cm above the right atrium using radiologic control and is then secured in this position and the skin wound closed. The wound is sutured with 4 O Dexon and the whole site sealed with a single strip of Opsite which does not require to be changed until the wound has healed.

The proximal end of the catheter is connected to a three-way tap which allows continuous intravenous infusion and monitoring of central venous pressure. Blood for a variety of investigations may be collected from the tap, and the technique employed is to precede specimen collection by the withdrawal of 20 ml of blood and to return this to the patient before recommencing the infusion.

**Discussion**
This simple technique provides practical and reliable venous access for periods in excess of 6 weeks and necessitates a minor surgical procedure for introduction of the catheter. The operation requires an experienced surgeon and approximately one hour for its completion and has a high degree of patient acceptability, particularly when compared to the alternative of multiple venepuncture. Furthermore, it avoids the hazards associated with repeatedly breaching the skin, thus reducing the risk of introducing exogenous organisms into the circulation with the development of septicaemia in the chronically ill and immunocompromised patient. This method offers substantial advantages over percutaneous introduction of a central venous line where thrombocytopenia may result in haemothorax: 2 recent patients
required platelet transfusion and pleural aspiration for this complication. Because of the position of the catheter the patient's arms are free for physiotherapy and for any manoeuvres that the individuals may wish to perform for themselves. For these reasons we consider that the investment of time, with the benefits for patient management, is more than justified.

In those individuals where continued intravenous manipulation is anticipated, the simultaneous creation of an arteriovenous fistula or insertion of a plastic prosthesis under the same general anaesthetic has become our recent routine practice, and markedly reduces the difficulties experienced with venous access in subsequent management.

Maintenance of the line is of paramount importance and, provided the catheter is flushed through with 5 ml of physiological saline every 4 hours, no problem in maintaining patency has been encountered. The development of infected thrombi at the end of the tubing and occlusion have not yet been encountered, even on removal of the catheter. In the experimental situation, thrombus in the catheter can be gently aspirated or lysed using low-dose infusion of streptokinase. One thousand units of heparin in each 1000 ml fluid should further reduce the risk of clot formation, and while we have used this regimen its efficacy has not been tested under controlled circumstances.

A point of particular importance is the ability to sample blood directly from the centrally-placed catheter. While it might be anticipated that differences would occur between blood collected from this plastic line and from clean puncture of a peripheral vessel, numerous comparisons of electrolytes, biochemical profiles, haematologic tests and blood cultures have consistently failed to show any difference between samples obtained from the two sites. The ability to avoid repeated venepunctures is considered a major benefit by patients who have personally experienced both approaches. It is unlikely that this procedure is new, but we feel that its redescription is justified at a time when an increasing number of patients are undergoing intensive chemotherapy and bone marrow transplantation, and are thus dependent upon reliable venous access for long periods of time.

Summary
A simple method of creating dependable long-term venous access is described. This method is suitable both for infusion and repeated blood sampling. Complications have thus far not been encountered.

Acknowledgment: We are grateful to Jeanne Walker for the illustrations.

References
INTRODUCTION

Bone marrow transplantation is the preferred form of treatment for patients with severe acute aplastic anaemia\(^1\) and immunodeficiency disease\(^2\). The procedure has also been used in the treatment of patients with refractory or relapsed acute leukaemia and may result in further disease-free periods in excess of two years in approximately 15% of such individuals\(^3\). On the basis of the latter experience bone marrow transplantation is now being evaluated in patients with acute leukaemia who have achieved their first complete remission. Preliminary data both for lymphoblastic\(^4\) and myeloblastic\(^5\) variants suggest that such an approach may hold advantages over cytotoxic maintenance programmes. Other possible indications for this procedure, while still controversial, include lethal genetically transmitted diseases such as thalassaemia major, constitutional or familial bone marrow failure, and chronic granulocytic leukaemia. Understandably, the morbidity and mortality that currently attend marrow transplantation are factors limiting its wider clinical use.

Two immunologic barriers influence the outcome of any transplantation procedure. The first of these is the satisfactory acceptance of the graft; failure to engraft is designated rejection. Best results are obtained by matching donor and recipient at the major histocompatibility complex. Despite HLA compatibility and mixed lymphocyte culture non-reactivity, problems may arise because of isoinmunisation of the recipient by prior administration of blood products. It is for this reason that transfusions should be minimised, ideally by early referral of patients. To encourage engraftment, the immunologic competence of the recipient is suppressed by drugs, such as cyclophosphamide, with or without whole-body radiotherapy. This preparative treatment is associated with destruction of residual haematopoietic tissue and thus contributes to the pancytopenia that characterises the post-transplantation period, when severe infection and thrombocytopenic bleeding are common problems.

Secondly, morbidity and mortality may result from graft-versus-host disease or reverse rejection. Here, an inflammatory reaction is directed predominantly against the cells of the gastrointestinal tract, the liver, and the skin, but its pathogenesis is controversial\(^6\). Because it is rare in identical twins or syngeneic transplants, importance is attached to optimal matching of donor and recipient. Nevertheless, the clinical syndrome, which may be either acute or chronic, occurs in up to 70% of apparently perfectly matched transplantation pairs. A prominent component of this syndrome is infection, where allogeneic granulocyte support may be life-saving.

The steady improvement in results of bone marrow transplantation must take into account the importance of specialised nursing and, particularly, the role of an efficient cell support section. The latter is needed to provide, on a moment-
to-moment basis, adequate numbers of functionally viable white cells and platelets. It is this facet of the programme that is illustrated by experience derived from five recently transplanted patients.

CASE REPORTS

Case No 1

POOR PROGNOSIS ACUTE LYMPHOBlastic LEUKAEMIA

A 10-year-old boy presented with a 12-day history of fever and headache, undue fatigue, and petechiae in the 24 hours before admission. Physical findings were limited to firm enlarged lymph nodes in the neck, axilla, and groin, and 4 cm hepatosplenomegaly. Initial haematology showed a haemoglobin of 9.8 g/dl; total white cell count of 114 x 10⁹/l, all lymphoblasts; and a platelet count of 60 x 10⁹/l. The biochemical profile was normal. Bone marrow examination confirmed the diagnosis of acute lymphoblastic leukaemia (FAB type L1). Lumbar puncture revealed the presence of numerous lymphoblasts in the cerebrospinal fluid. Radiology of the dorsal spine showed mild osteoporosis and vertebral collapse without positive evidence of leukaemic infiltration.

Complete remission was achieved successfully with a combination of vincristine, prednisone, 6-asparaginase and Adriamycin. The central nervous system was cleared of leukaemic blasts with twice weekly alternating intrathecal cytosine arabinoside (30 mg/m²) and methotrexate (12 mg/m²). Two courses of consolidation chemotherapy were given using the four induction drugs. Standard craniospinal therapy was given without complication.

In view of the bad prognosis predicted by the high circulating blast count and the presence of central nervous system disease, the patient and his family were offered bone marrow transplantation in this first complete remission. An HLA identical and MLC non-reactive 12-year-old brother acted as donor after informed consent had been obtained.

Following placement of a central venous catheter the patient was prepared with cyclophosphamide, 60 mg/kg, administered by intravenous infusion on two consecutive days with attention to fluid balance and electrolyte status. Following a 24-hour rest period total body irradiation was given to a mid-plane dose of 1.000 rads; the skull was shielded at 500 rads to compensate for previous administration of 3.200 rads cranial irradiation.

Under general anaesthetic 440 ml of marrow was collected from the donor by multiple percutaneous punctures from the sternum and both iliac crests. 2.3 x 10⁸ nucleated cell/kg were infused into the recipient. The adequacy of the donor marrow was confirmed by in vitro bone marrow culture. The donor was discharged from hospital 24 hours after marrow donation.

In the post-transplantation period the patient received daily parenteral intravenous alimentation. For five days following infusion of the marrow the donor buffy layer was infused providing both white cells (mean of 2.2 x 10⁹; range 0.7 — 4.0 x 10⁹), and platelets (mean of 0.4 x 10¹¹; range 0.2 — 0.8 x 10¹¹).

Because the patient remained pyrexial despite an intravenous antibiotic regimen of cephaloradin, aminoglycoside and metronidazole, allogeneric white cell support was continued until day 14 when the temperature returned to normal.

Successful engraftment was confirmed by a rising peripheral platelet and granulocyte count on day 12. Trephine biopsy was hypocellular but all lines were represented.

Comments

The immediate post-transplantation course in this patient was characterised by mild to moderate graft-versus-host disease with persisting skin rash and transient diarrhoea requiring intravenous hyperalimentation and corticosteroids. There are residual Cushingoid striae but no measurable endocrine abnormalities. The osteoporosis, perhaps due to leukaemic infiltration, has resolved with calcium and vitamin D supplementation.

A transient period of jaundice was unexplained and may have been the result of viral infection from transfusion therapy, a reflection of graft-versus-host disease, or incidental infectious hepatitis. One year from transplantation the patient again had elevation in liver enzymes and mild conjugated hyperbilirubinemia. There were no demonstrable antibodies against hepatitis A or B, and the possibility of intermittent biliary tract obstruction was considered but not proven.

The patient has remained immunocompromised with hyperglobulinaemia and has received parenteral gammaglobulin. In addition, he has had an episode of staphylococcal septicaemia requiring six weeks of intravenous antibiotics. Recovery was uneventful. One year after being transplanted, he developed an acute abdomen requiring emergency laparotomy. Pigment gallstones obstructing the common bile duct were found. The patient handled wound healing and his surgery without difficulties.

At the present time, one and a half years after successful bone marrow transplantation and despite a variety of complications, he is disease-free and has mild (Grade I — II) but steady resolving graft-versus-host disease.

Case No 2

POOR PROGNOSIS ACUTE UNDIFFERENTIATED LEUKAEMIA

A 22-year-old student presented with a one week history of anaemia and bleeding from his gum margins. Physical examination was negative apart from 1 cm hepatosplenomegaly. Initial haematology showed a haemoglobin of 12.5 g/dl; total white cell count of 35 x 10⁹/l; 11% neutrophils, 5% eosinophils, 1% basophils, 38% lymphocytes (many of which were atypical), 6% monocytes, 3% promyelocytes, 5% myelocytes, 31% metamyelocytes; platelet count was 146 x 10⁹/l. The biochemical profile was normal. Bone marrow examination showed marked hypercellularity and many ringed sideroblasts. The marrow was extensively involved with undifferentiated acute leukaemia.

Initial attempts to induce remission in the patient with a combination of an anthracycline antibiotic, MARCH 1983
cytosine arabinoside and the epipo-
dophyllotoxin VP 16-213 were un-
successful. Remission was subse-
sequently achieved with the combina-
tion of vincristine, prednisone, e-
asparaginase and adriamycin. Fol-
lowing two courses of consolidation the 
patient was offered bone 
marrow transplantation in view of 
the poor prognosis associated with 
acute undifferentiated leukaemia. 
Conditioning was with the stan-
dard regimen of cyclophosphamide 
and total body irradiation12. He 
received 4 x 10⁸ nucleated cells/kg 
from an HLA identical MLC non-
reactive brother. The adequacy of 
the donor marrow was confirmed by 
_in vitro_ bone marrow culture. 
Post-transplantation stem cell boost-
ing was provided with adminis-
tration of buffy layer for five days. 
Mean white cell yield 3.4 x 10¹⁰ 
(range 1.7 - 3.8 x 10¹⁰). Mean 
platelet yield 6 x 10¹⁰ (range 0.5 
- 1.2 x 10¹¹). No further cell support 
was needed.

Comments 
Mild to moderate graft-versus-host 
disease developed which responded 
dramatically to 1.5 mg/kg 
parenteral prednisone/24 hours and did 
not reappear when the cortico-
steroid was withdrawn. The patient 
developed an acute abdomen with 
radiologic and ultrasonographic evi-
dence of acute biliary tract obstruc-
tion. He rapidly developed fulmi-
nating sepsicaemia and died before 
surgery could be undertaken.

At post mortem biliary obstruc-
tion had occurred as a result of a 
mucosal cast having impacted and 
totally obstructed the common bile 
duct. No residual evidence of leu-
kaemia was demonstrated and bone 
marrow engraftment was con-
firmned.

Case No 3

**SEVERE ACUTE APLASTIC ANAEMIA**

A 35-year-old woman was referred in 
extremis after having been ill for 
six weeks. There had been bruising 
and minor trauma with severe head-
ache and vaginal bleeding for four 
weeks. Examination showed ecchym-
moses around both eyes and exten-
sive purpura was present. There 
was marked neck stiffness and the 
patient was stuporous although 
moving all her limbs. Pupils were 
equal and responsive. No other 
focal or neurologic signs were pres-
ent. Lumbar puncture showed 
bloodstained cerebrospinal fluid.

Initial haematology showed a 
haemoglobin of 2.7 g/dl; white cell 
count of less than 1 x 10⁸/l and platelets less than 10 x 10⁹/l. Only 
lymphocytes were circulating in the 
peripheral blood. The biochemical 
profile was normal. Bone marrow 
aspiration and the trephine biopsy 
showed aplasia.

The patient was initially managed 
conservatively from the standpoint 
of her sub-arachnoid haemorrhage 
with irradiated allogeneic platelet 
transfusions. She responded well to 
this management and once fully 
conscious and able to make deci-
sions accepted bone marrow trans-
plantation. A central venous line 
was placed and conditioning under-
taken with a standard regimen of 
cyclophosphamide. Total body ir-
radiation was not used.

The patient was uneventfully 
transplanted from her HLA identi-
cal and MLC non-reactive sister, re-
ceiving 3.8 x 10⁸ nucleated cells/kg 
and the graft viability was estab-
lished by _in vitro_ marrow culture.

The patient received five days 
post-transplantation buffy layer to 
supplement haematopoietic stem 
cells; mean white cell yield 1.2 x 10¹⁰ 
(range 0.5 - 2.3 x 10¹⁰) and pla-

teelets with a mean of 1.0 x 10¹¹ 
(range 0.5 - 1.9 x 10¹¹). Engraft-
ment was demonstrated by rising 
peripheral count confirmed by tre-
phine biopsy on the 12th post-trans-
plant day.

On the 11th post-transplant day, 
after buffy layer had been discon-
tinued, the patient was again pyre-
xial and had an episode of fresh 
rectal bleeding. Allogeneic white 
cells and platelets were recom-
mented and the patient was unex-
pectedly found dead in bed on the 
17th post-transplantation day.

Comments 
The pyrexial episode was investi-
gated with blood cultures which 
failed to grow an organism. Never-
theless, the standard antibiotic regi-
men of cephalosporin, aminoglyco-
side and metronidazole were com-
menced and because the fever per-
sisted allogeneic white cells were 
infused.

The cause for the rectal bleed-
ing was never established and the 
patient remained thrombocytopeni-
at this stage and there was a prompt 
response to platelet infusion.

Post mortem examination failed 
to reveal the cause of death. No 
cerebral bleeding was demon-
strated. Marrow engraftment was 
confirmed.

Case No 4

**FANCONI ANAEMIA**

A diagnosis of Fanconi anaemia 
was made at the age of 12 when the 
patient was seen with easy bruising 
and thrombocytopenia. During the 
ensuing years these symptoms were 
moderately controlled on low doses 
of corticosteroids. In the year 
preceding his referral he had failed to 
respond to anabolic androgens and 
was developing an increasing blood 
transfusion requirement. On admission 
he was pale and tachylic. Scattered 
purpura and ecchymoses were 
present. He was markedly Cush-
ingoid.

Initial haemoglobin was 4.3 g/dl; 
total white cell count 4.8 x 10⁹/l 
with 22 % neutrophils; platelet 
count was 3 x 10⁹/l. Bone marrow 
showed erythroid hypoplasia with 
marked megaloblastic change, 
normal granulopoiesis, and scanty 
ogakaryocytes. In view of the 
rapid deterioration and despite the 
presence of a strong multispecific 
antibody, the patient was condi-
tioned with a standard regimen of 
cyclophosphamide and transplanted 
from an HLA identical and MLC 
non-reactive sister.

Particularly in view of previous 
sensitisation, importance was at-
tached to the buffy layer support 
which yielded a mean white cell 
count of 1.3 x 10⁹ (range 0.5 - 3.6 
x 10⁹) and a mean platelet count of 
0.5 x 10¹¹ (0.5 - 1.4 x 10¹¹).

In the two weeks following 
transplantation, most prominently etweeen days 10 and 14, the patient 
developed severe mucositis, diar-
rhoea and skin desquamation. His 
hands became swollen and deep 
bullous lesions developed over the 
knuckles. These cutaneous findins

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were associated with the isolated biochemical abnormality of a rising total and conjugated bilirubin.

This clinical syndrome was interpreted as being due to acute graft-versus-host disease and the patient treated with high doses of corticosteroids.

Despite the obvious progression of this immunologic syndrome, engraftment was demonstrated on the 12th post-transplant day by rising granulocyte and platelet count. Confirmation was obtained by bone marrow aspiration and trephine biopsy.

There was rapid progression of the graft-versus-host disease and by the 21st post-transplant day it was progressing to scleroderma. On the same day there was a dramatic temperature rise and the patient died from fulminating septicaemia superimposed upon his graft-versus-host disease.

Autopsy confirmed the presence of engraftment and extensive graft-versus-host disease.

**Comment**

Bone marrow transplantation in patients with constitutional anaemia, as exemplified by the Fanconi syndrome, remains controversial although successful cases have been reported. The uncertainty rests upon an inability to ensure that the donor is not also affected by the same chromosomal abnormality as the patient. In the present case appropriate studies excluded this possibility although more subtle lesions may have existed and escaped attention by available techniques.

This patient exemplifies the situation where post-transplantation stem cell boosting with buffy layer has been most encouraging. In individuals sensitised from previous exposure to blood or components there is limited evidence that immune rejection may be compensated for by buffy layer infusion. Clearly, at least in the short term, the transplantation was successful in re-establishing haematopoietic function.

The acute graft-versus-host disease is believed to represent direct transmission of sensitised lymphocytes from donor to recipient in the marrow graft. The onset and the clinical course are typical of the acute variant of graft-versus-host disease and, despite the administration of high-dose corticosteroids, the course followed by this patient is in keeping with that described by other more experienced investigators.

**Case No 5**

**SEVERE ACUTE APLASTIC ANAEMIA**

A 25-year-old female presented with a one-month history of lower abdominal pain which was diagnosed as salpingitis and treated with an unknown antibiotic by her private physician. Shortly thereafter she experienced epistaxis and menorrhagia, followed by increasing dyspnea, palpitations, and a sharp decline in effort tolerance. Apart from the antibiotics there was no history of drug ingestion. Physical examination was normal except for bilateral fundal haemorrhages.

Initial laboratory findings included a haemoglobin of 6.0 g/dl; white cell count 1.5 x 10^9/l, with 88% lymphocytes; and platelet count of 13 x 10^9/l. The biochemical profile and chest X-ray were normal. Bone marrow aspirate and trephine biopsy showed aplasia. Following conditioning with cyclophosphamide, the patient was transplanted from an HLA identical and MLC non-reactive brother, followed by five days post-transplantation buffy layer administration. The mean white cell yield was 2.5 x 10^10 (range 0.9 — 3.7 x 10^10) and mean platelets were 1.0 x 10^11 (range 0.3 — 1.8 x 10^11).

In the second week low grade fever continued despite appropriate antibiotics and in view of the pelvic inflammatory disease with which the patient presented, allogeneic granulocyte support was continued.

By day 13 the temperature had settled and engraftment confirmed by bone marrow aspirate and trephine biopsy. All support was discontinued from the end of the second post-transplantation week.

**Comment**

This patient developed rapidly increasing weakness ascribed to the administration of epsilon aminocaproic acid which she received in large amounts for her menorrhagia. Her course was further complicated by the development of septicaemia, and death resulted from sub-arachnoid haemorrhage.

**CELL SUPPORT**

The machine used for the collection of buffy layer is the NCI-IBM 2990 or, more recently, the 2997, both of which operate on the principle of differential centrifugation. The essential difference between the two machines is that the newer NCI-IBM 2997 has a disposable bowl so that risk to individuals on the machine from infection is no longer present. The blood leaves an arm vein of the donor and enters a slowly spinning centrifuge bowl where the white cells and platelets are segregated from the red cells and may be selectively removed, with all other components being returned to the donor.

In the NCI-IBM 2990 the polycarbonate bowl is primed with 1 000 ml of 0.9% saline containing 5 000 units of heparin. Following cannulation of the patient a further 5 000 units of heparin are administered as an intravenous bolus. A centrifuge speed of approximately 600 rpm is used and flow rates vary between 30 and 50 ml/minute. Under these circumstances the leucocyte and buffy layer is evident as a yellow band situated between the red cells and the plasma, and is visible through the transparent perspex deck of the centrifuge bowl. Extraction of white cells and platelets takes place at a rate between 3 and 6 ml/minute, and between 150 and 300 ml is usually collected from each donor.

Donations require informed consent and predonation full blood counts are routinely obtained on each occasion. Donor temperature, blood pressure and pulse are monitored throughout the course of the procedure. The donor, in addition to having given marrow, is routinely used in the five days immediately following transplantation with the theoretical intention of enhancing the number of haematopoietic stem cells administered to the recipient.

In those patients where fever and granulocytopenia, often associated with thrombocytopenia, are en-
countered, a further 10 to 14 days
donation of buffy layer is possible
from the same donor without either
undue difficulty or stress. If the
original volunteer is unable to con-
tinue donations while the recipient
is still dependent upon allogeneic
cells, ABO compatible relatives
and unrelated donors are used.
Under these circumstances the gra-
nuocytes are irradiated to 1500
rads to prevent proliferation of lym-
phoid cells which might produce or
enhance graft-versus-host disease.

A number of manipulations can
be used to increase the yield of
buffy layer collections, including
corticosteroid administration to the
donor and addition of hydroxyethyl
starch to the venous line supplying
blood to the bowl. The latter sub-
stance may cause headache due to
volume expansion, decrease in haem-
ogloin and platelets due to hae-
modilution in the donor, and pruri-
tis.

In contrast to differential cen-
trifugation the alternative technique
of continuous-flow filtration may be
used for granulocyte collection.
However, only centrifugation is suit-
able for buffy layer collection since
stem cells, lymphocytes, platelets,
and granulocytes are collected by
this technique.

Where filtration is used, high gra-
nulocyte yields may be obtained but
there is recent evidence that the
cells obtained are functionally and
ultrastructurally inferior to those
recovered following centrifugation.

In order to obtain cells by filtra-
tion heparinised blood is passed
over nylon filters at flow rates be-
tween 40 and 100 ml/hour. Granu-
locytes adhere to the filter and re-
covered by elution with a citrate-
containing solution. Other cellular
components do not remain in the
filter. Because a volume in excess of
10 litres needs to pass over the filter
donations usually require two
hours. A recent modification has
been the use of in-line elution but
this remains largely developmental.
It has, however, been suggested,
but not yet proven, that both cell
yield and function are significantly
improved by this approach.

During elution granulocytes can
be dislodged from the filter by per-
cussion of the casings and cells then
concentrated by centrifugation at
1600 x g for 20 minutes. The final
volume infused varies between 100
and 300 ml, and should take place
within one hour of collection.

Complications occur with both
techniques. With centrifugation
there may be a drop in haemoglobin
which requires correction by admin-
istration of fresh irradiated packed
red cell. Thrombocytopenia which is
transient occurs rarely. In a number
of patients venous access may be
difficult, requiring the use of arterio-venous shunts. Clotting
may occur in the extracorporeal cir-
culation due to insufficient use of
anticoagulant but this should not be
encountered when experienced
staff operate the machine. Similar-
ly, anxiety occurs in some donors
but can be overcome by the presen-
tce of experienced and competent
nursing and medical staff.

The complications arising from
haemofiltration also include diffi-
culties with venous access and clot-
ting. However, here an abdominal
pain syndrome is recognised which
is believed to reflect complement
activation.

BONE MARROW TRANS-
PLANTATION PROCEDURE

Donors commence oral iron and
folicate supplementation two weeks
before transplantation. One unit of
whole blood is collected in the week
before transplantation. The buffy
layer is separated and administered
to the recipient 24 hours before
commencement of the cyclophos-
phamide conditioning regimen. The
red cells from the same unit are re-
tained for re-transfusion to the
donor during the subsequent collect-
ion of bone marrow.
Donor and recipient are compat-
ible at the major loci within the
human histocompatibility complex.
Ideally, there should be no blood
group incompatibility, although re-
duction in red cell antibodies to in-
consequential levels can be effected
by means of large volume plasma
exchange4.

Twenty-four hours after adminis-
tration of the buffy layer, which
theoretically triggers immunologi-
cally competent cells in the recipi-
ent into cycle and hence renders
them maximally responsive to cyto-
toxics, cyclophosphamide condi-
toning is commenced. In patients
with severe acute aplastic anaemia,
50 mg/kg is given by intravenous
infusion over half an hour on four
consecutive days. In leukaemic
patients undergoing first remission
bone marrow transplantation a
slightly different regimen is used; 60
mg/kg of cyclophosphamide is given
for two days, following which the
patient rests for 24 hours and then,
on the day of transplantation,
undergoes 1000 total body irradia-
tion at a rate of 9 rads/minute.
The object of these conditioning regi-
mens is to destroy all immunologi-
cally competent lymphatic tissue
which may be capable of mounting
a rejection response.

Under general anaesthesia, and
with informed consent, marrow is
collected from the donor by mul-
tiple punctures of the sternum and
both iliac crests. Between 1 and 3 ml
of marrow-rich blood are aspirated
from each puncture site and a final
volume of approximately 10 ml/kg
of recipient's body weight collected.
The aspirated material is anticoagu-
lated in heparin-containing culture
medium and converted to a mono-
cellular suspension by passing it
through a series of stainless steel
screens. The graft is infused over
half an hour to the recipient. Atten-
tion is given to any discomfort in
the chest which may be associated
with multiple small fat emboli. The
arterial oxygen saturation is obser-
vated at the same time.

Apart from experiencing some
discomfort donors are usually able
to leave hospital the next day and
have little trouble in attending the
Cell Support Section to donate
buffy layer on the separator.

The recipient is nursed in iso-
lation and receives buffy layer daily
for the first five days of the post-
transplantation week. All support is
then discontinued unless the patient
is pyrexial or has significant throm-
bocytopenic bleeding. In the face of
pyrexia, antibiotics (tobramycin,
cephamandole, and metranidazole)
are commenced while awaiting ap-
propriate cultures. Failure to lyse
fever within 24 hours is an indica-
tion for allogeneic white cells, with
an arbitrary level of 1.5 granulo-
cytes x 10^10 the daily goal.
IN VITRO BONE MARROW CULTURE

Culturing haematopoietic stem cells or committed progenitors in semisolid agar in the laboratory is a useful technique which may be applied to monitoring the adequacy of the graft being infused\(^5\). In principle, the marrow is cultured in a system that provides both the nutrients and the stimulating substances necessary for the growth and proliferation of the progenitor cells. The appearance of small cell aggregates can then be counted, thereby providing an index of the number of stem cells in the graft.

In practice, the stimulating substance is a glycoprotein which is elaborated and released into the agar. The marrow from the donor is washed and suspended in a second agar layer called the overlay in a 35 mm petri dish which is then incubated in a humidified atmosphere containing a fixed amount of carbon dioxide at 37°C. The appearance of clusters and colonies are determined at days 0, 3, 7, 10 and 12. By definition, clusters contain between 3 and 49 cells, white colonies have more than 50 cells present.

Under normal circumstances the earliest evidence of in vitro marrow growth is evident between day 3 and 7, but the optimal colony cluster ratio has been recorded in our laboratory at day 10. In each of the patients discussed in the case studies normal numbers of colonies and clusters were demonstrable at this time, establishing that committed haematopoietic stem cells, at least for the monocyte macrophage line, are present in normal numbers. Experience has demonstrated that such in vitro growth patterns correlate with the supply of adequate numbers of stem cells as reflected in uniform engraftment of patients where sensitisation has not previously taken place.

DISCUSSION

Bone marrow transplantation has become increasingly successful, especially in the last decade. This improvement reflects both a better appreciation of the need to match donor and recipient at the major histocompatibility complex and a clearer understanding of the immunobiology of the procedure, particularly in providing a sufficient number of haematopoietic stem cells to bring about marrow reconstitution. Nevertheless, as these two important variables have been managed, new challenges have emerged.

It has become apparent that prior exposure to blood or blood group antigens may lead to isoinmunisation in the recipient and subsequent graft rejection. Accordingly, patients being considered for transplantation should be referred early to a transplantation centre so that this essentially iatrogenic complication may be avoided.

A second source of failure now amenable to treatment is that of thrombocytopenic haemorrhage. With the steady improvement of technique for allogeneic platelet transfusion, this complication has diminished in importance. Regrettably, this advance may be a two-edged sword in that platelet transfusions may be used indiscriminately in the short term in the hope that conservative therapy may allow spontaneous marrow recovery to occur, particularly in patients with severe acute aplastic anaemia. In this situation the problems of isoinmunisation are enormous. Platelet survival may be shortened and the option of transplantation may be lost by inadvertent stimulation of antibody production directed against donor cells.

With control of thrombocytopenic haemorrhage, infection, usually bacterial, has taken over as the major cause of morbidity and mortality in the transplant patient. Two major factors contribute to the propensity to infection. Firstly, in patients with severe acute aplastic anaemia, severe granulocytopenia is part of the disease and many of these patients reach the transplant centre with established infection, commonly of the respiratory tract. Secondly, the conditioning regimens aimed at diminishing host immune competence in order to facilitate graft acceptance superimpose immune destruction of the host upon pre-existent granulocytopenia.

Since engraftment should not be a problem in properly selected donors who have not been previously isoinmunised, it follows that carefully selected allogeneic platelet and granulocyte support occupies a crucial role in determining the success of any marrow transplantation.

The infusion of buffy layer to boost the number of stem cells given to the patient is theoretically sound. However, recent evidence suggests that this particular manoeuvre is less helpful than previously anticipated, particularly if adequate numbers of stem cells are collected in the initial graft.

Nevertheless, the provision of white cells and platelets during the first week following transplantation has notably improved the general condition of the patients. Until such time as a randomised prospective study becomes available, the advantages are sufficiently attractive for us to continue with this procedure.

Of a less controversial nature is the use of allogeneic granulocyte support in the patient who is pyrexial and pancytopenic following transplantation. Despite suggestions that the fever may be related to neutropenia per se rather than infection\(^6\), and the suggestion that prophylactic granulocyte transfusions are not helpful, our own experience has been otherwise. It remains to be determined by prospective randomised study whether the provision of adequate numbers of functionally intact granulocytes to the infected neutropenic patient following marrow transplantation can be safely omitted.

CONCLUSION

It is concluded from recent experience that transfusion of buffy layer containing granulocytes and platelets, both routinely in the week following transplantation and subsequently for the indications of fever and haemorrhage, is beneficial in the bone marrow transplant recipient. We emphasise the need to maintain rigid quality control in number and function of cells transfused, to recruit an adequate donor panel, and to maintain a highly trained nursing staff to operate an active Cell Support Section as an in-
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The role of continuous-flow blood fraction separators in clinical practice

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The continuous-flow blood fraction separators have the capacity to selectively exchange large volumes of plasma or red cells with minimum risk or discomfort to the patient. These procedures are effective and their use in clinical practice is increasing, as experience with 5 recent cases illustrates.

Myasthenia gravis is an antibody-mediated neuromuscular disease. Normally the locally released acetylcholine from the nerve occupies a specific receptor site on the motor end-plate, resulting in muscular contraction. In myasthenia gravis an antibody is formed and blocks the receptors so that the contractile response to a given amount of acetylcholine is suboptimal. The rationale for plasmapheresis in this condition is to reduce the quantity of the acetylcholine receptor antibody present in the circulation. Simultaneously, immunoglobulin production is decreased by administering immunosuppressive drugs. Although of greatest benefit in the short term, prolonged periods of plasma exchange may occasionally be needed.

In the pregnant woman iso-immunized to rhesus antigen, it is logical to anticipate that lowering the anti-Rho (D) titre in the maternal serum will reduce the severity of the haemolytic disease in the fetus. In those individuals who have not been given prophylactic immunoglobulin and who have become sensitized, plasmapheresis is a practical approach to resolving this problem.

Bone marrow transplantation depends upon compatibility between donor and recipient. In unusual circumstances HLA identity and MLC non-reactivity are associated with blood group incompatibility. This dilemma can be resolved by continuous-flow plasma exchange in which naturally occurring antibodies directed against the ABO system are greatly decreased. Any residual antibody is adsorbed by infusion of suitable red cell antigen prior to grafting.

Under normal circumstances adult red cells contain predominantly HbA which effectively delivers oxygen to metabolizing tissue. In sickle cell disease this HbA is replaced by an abnormal haemoglobin (HbS). The presence of HbS results in the defective red cells called sickle cells, which deform and may block the microcirculation when oxygen tension is lowered. Serious complications with tissue infarction may then arise. Since general anesthesia and major surgery may be associated with varying degrees of hypoxia, it is rational to protect the patient from these risks by pre-operative red cell exchange in which normal levels of HbA are substituted for the defective haemoglobin.

Five case reports are presented to illustrate the increasing use of continuous-flow centrifugation in current clinical practice.

Blood fraction separator procedures and laboratory techniques

Plasmapheresis

Continuous-flow exchanges were carried out using the National Cancer Institute - International Business Machine (NCI - IBM 2990) Blood Fraction Separator. A vein-to-vein technique was employed, the blood passing through a special bottle where separation of the various components takes place in response to centrifugal force. Two patients required the insertion of an arteriovenous shunt to ensure adequate vascular access. Before commencement of the procedure the 250 ml dead-space present in the lines and the centrifuge bowl was primed with a solution of physiological saline and heparin (100 mg/kg).

Effective plasma exchange is complete between 90 and 120 minutes with centrifuge speeds of 1000 rpm and blood flow rates between 25 and 50 ml/min. Ideally, exchange of 75 ml/kg or twice the estimated plasma volume is attempted, but this is influenced by many variables including patient tolerance and the kinetics of exchange...
between intravascular and extravascular spaces for the substance being removed. In the first 3 patients an exchange of 2 litres was achieved on each occasion. Priming anticoagulation was with 5000 IU heparin given as an intravenous bolus at the time of cannulation. The replacement fluid consisted of an electrolyte solution containing 20% salt-poor human albumin. During the procedure a trained nurse was present at all times.

In the patient undergoing anti-A depletion, two separate but longer procedures were employed in which 6 and subsequently 14 further litres of plasma were exchanged for ABO plasma.

**Red cell exchange**

The technique is essentially similar to that used for plasmapheresis. A volume of 2.825 ml of the patient's blood containing high concentrations of HbS was exchanged for an equivalent volume of normal red cell concentrate with a packed cell volume of 80%. The exchange procedure lasted 3½ hours and was performed at a flow rate between 10 and 15 ml/min.

**Acetylcholine receptor antibody titre determinations**

Titres of the antibody directed against the acetylcholine receptor were determined just before each plasmapheresis. An assay was used in which competition between antibody and radio-labelled α-bungarotoxin is determined for receptors present on eel cells. In this assay antibody concentration in the patient's plasma is inversely related to binding of labelled toxin to the cells.

**Estimation of rhesus (D) antibody levels**

These were determined by the indirect antihuman globulin (AHG) technique and estimations of specific anti-Rho (D) antibody concentration were made using a reference serum (National Institute for Biological Standards and Control, London).

**Estimation of anti-A titres**

Iso-agglutinin anti-A titres were determined against A+ and A- cells at 22° and 4°C. Immune anti-A was titrated against A+ cells in saline, bromelin and AHG.

**Haemoglobin measurements**

A venous blood sample was obtained before red cell exchange and at regular intervals throughout the procedure. The Hb level was determined on the Coulter Counter Model S. Red cell lysates were electrophoresed on cellulose acetate and quantitated by integrating densitometry.

**Case report and results**

**Case 1**

In May 1976 a 56-year-old woman presented with drooping eyelids, weakness of the neck and difficulty in swallowing. A response to an intravenous injection of short-acting anticholinesterase (Tensionil) established the diagnosis of myasthenia gravis. An acceptable level of skeletal muscular activity was maintained with proximine 15 mg 3 times a day, and prednisone 10-40 mg on alternate days. Chest radiography and mediastinal tomography suggested a thymoma, and thymectomy was performed 1 month after diagnosis. The histological examination of the tumour showed a mixed cell thymoma with a predominantly epithelial component.

Two days postoperatively the patient had a sudden respiratory arrest requiring tracheostomy and intermittent positive pressure ventilation for 1 week. Subsequent recovery was uneventful, and she was discharged from hospital 1 month later on 20 mg prednisone on alternate days, and 15 mg neostigmine and 60 mg pyridostigmine (Mestinon) 3 times a day. After a further month of this treatment the patient's condition started to deteriorate with gradually increasing weakness in the extremities progressing to respiratory failure, again necessitating tracheostomy and intermittent positive-pressure ventilation. Tensionil tests at intervals showed a slight improvement in some muscle groups but respiratory insufficiency remained a problem. Anticholinergic drugs and steroids were adjusted according to muscle weakness.

A decision to commence plasmapheresis was made 8 months after the initial diagnosis of myasthenia gravis. Intensive daily 2-litre plasma exchanges via an arteriovenous shunt were carried out for 2 weeks, during which time immunosuppressive therapy with 50 mg azathioprine and 50 mg cyclophosphamide a day was commenced. The patient (50 kg body weight) showed clinical improvement only after the 6th week of this regimen, reflected in steady increase in the strength of respiratory and neck muscles, with concomitant increase in vital capacity.

Acetylcholine receptor antibody titres were monitored (Fig. 1). It can be seen that the pretreatment level between 39 and 54 x 10^9M reverted to a stable post-plasmapheresis level of approximately 4 x 10^9M.

![Fig. 1. Response in acetylcholine receptor antibody titre to plasmapheresis.](image-url)

An attempt to reduce maintenance immunosuppressive therapy was unsuccessful, leading to recurrence of clinical signs and symptoms. Increase in anticholinergic drugs, immunosuppressive therapy and repetition of plasmapheresis were again successful. The patient was eventually discharged home 11 months after her second relapse on maintenance doses of 50 mg azathioprine daily,
60 mg pyridostigmine 4 times a day, and 30 mg prednisone on alternate days with weekly plasmapheresis. The plasmapheresis was gradually reduced and was discontinued 7 months later.

At the present time the patient leads a normal life with only minor weaknesses in the bulbar muscles, reflected in nasal speech.

Case 2

A 60-year-old woman developed bulbar symptoms with dysphonia and dysphagia 2 years before referral. The diagnosis of myasthenia gravis was confirmed on testing, and mediastinal tomography showed a tumour mass which was uneventfully resected. Histological examination showed a benign thymoma. Transient clinical response followed the operation and relapse was controlled with anticholinesterase drugs.

Eight months before the present admission further deterioration necessitated corticosteroids (prednisone 1 mg/kg) with short-lived improvement in bulbar symptoms. In the previous 6 months there had been steady deterioration, with marked change in speech and inability to eat. All nutrition was provided by means of nasogastric intubation. On examination there was prominent nasal speech and classic muscle fatigue on repetitive movements.

In the face of a refractory and progressive bulbar myasthenia, prednisone (0.5 mg/kg/24 h) and azathioprine (2 mg/kg/24 h) were added to the established cholinesterase regimen, and plasmapheresis against fresh frozen plasma was commenced 3 times a week. The response was dramatic. The speech started to revert to normal after the second 2-litre exchange and within 1 week the patient was able to swallow normally and eat solid foods.

Comment

In patients with myasthenia gravis the elaboration of an antibody directed against acetylcholine receptors on the motor end-plate interferes with normal muscular function and is thus considered to arise on an auto-immune basis. The first essential step is to remove any mediastinal tumour that may exist since therapy is less satisfactory in patients with a thymoma. Attempts to prolong the survival of biologically produced acetylcholine by giving cholinesterase inhibitors remains the standard form of medical management. In patients where this is unsuccessful, and, particularly where antibody can be demonstrated in plasma, immunosuppressive regimens combining corticosteroids and either azathioprine or cyclophosphamide are rational third lines of treatment. When these measures are unsuccessful, intensive plasmapheresis to remove a percentage of the antibody has recently been introduced. Most of the experiences suggest that the greatest benefit is found in the early stages of treatment while immunosuppressive drugs are producing their effect. This was clearly the case in the one patient, but the good response may have been due to immunosuppressive drugs, plasma exchange or both. From experience with the other patient it appears that not only is plasmapheresis now an important form of treatment in the patient with refractory myasthenia gravis, but, notably, considerable periods of time may elapse before clinical improvement becomes manifest.

It is therefore clear that while many patients will respond to early intensive plasmapheresis, failure to achieve objective improvement within the first 4-6 weeks should not be considered a contraindication to maintaining an intensive programme of plasma exchange for more prolonged periods.

Case 3

A rhesus (D)-negative woman with a history of 2 previous pregnancies presented in June 1979 at 26 weeks’ gestation. The first pregnancy in 1968 had been uncomplicated and a normal infant was delivered at 40 weeks showing no signs of jaundice. Following this pregnancy, anti-Rho (D) gammaglobulin was not administered. The latter procedure is now standard practice to prevent Rh iso-immunization. During the second pregnancy increased levels of anti-Rho (D) antibody were detected. This infant was delivered by caesarean section at 38 weeks and required two exchange transfusions.

On presentation with the third pregnancy the anti-Rho (D) titre was 1:256 and amniocentesis showed a raised optical density density (ODD) level of 0.544, which is in the upper zone of Liley’s graph. The serum bilirubin level was 9.4 µmol/l, and serum protein was 5.5 g/l. These findings suggested a severely affected fetus.

Plasmapheresis began at 27 weeks, when the anti-Rho (D) titre was recorded at 1:256 by the antiglobulin technique. On alternate days plasmapheresis (50 ml/kg) was performed, with an effective reduction in antibody titre (Fig. 2). A caesarean section was performed at 32 weeks’ gestation with no complications in the mother. The infant required four exchange transfusions and the total serum bilirubin level rose to 350 µmol/l. During each exchange transfusion this level was lowered. Thirty-six hours after birth the infant developed respiratory distress and required intubation and ventilation with positive pressure for 104 hours. No further complications occurred, the infant commenced oral feeding on the 42nd day and continued to improve until it was discharged on the 58th day.

![Fig. 2. Anti-Rho (D) titre before and after plasma exchange.](image)
Comment

Rhesus iso-immunization, particularly when high antibody levels are present in the mother, is a major risk factor for neonatal jaundice. To prevent the development of hydrops fetalis delivery would be required at a time during gestation when the fetus is at risk from the respiratory distress syndrome. In the normal course of events iso-immunization is prevented by the administration of anti-Rho (D) immunoglobulin in any situation where the mother is at risk of fetomaternal haemorrhage. Failure to deliver such prophylactic therapy with the subsequent development of rising titres in the mother has until recently presented a major challenge for the obstetrician. The availability of continuous-flow plasma exchange, as illustrated by this case, is an effective means for the management of this clinical syndrome.

Case 4

A 15-month-old girl presented with frequency, dysuria and fever. A diagnosis of acute pyelitis was made and treatment commenced with sulphonamides and streptomycin. Some improvement was evident after 3 days, but a week later the child was still listless and anorectic. Examination showed pallor of the mucous membranes, a firm, non-tender 4 cm splenomegaly, and a soft, non-tender hepatomegaly. The haemoglobin concentration was 9 g/dl and a diagnosis of haemolytic anaemia was made. Further investigation showed a reticulocyte count of 11%, and a serum bilirubin value of 42.5 μmol/l. Sickling of red cells was noted on a peripheral blood smear. Haemoglobin electrophoresis confirmed the presence of large concentrations of Hbs. During childhood two further crises arose after upper respiratory tract infections, each associated with a fall in haemoglobin levels. Further growth and development was normal.

At the age of 19 years the patient presented with upper abdominal pain. An oral cholecystogram confirmed the clinical diagnosis of cholelithiasis. An elective cholecystectomy was planned, and to prevent sickling of red cells during general anaesthesia and the operation, it was decided to use continuous-flow red cell exchange to reduce the Hbs level. Before this procedure, the haemoglobin level was 8 g/dl of which 2.3% was HbA, 85.3% HbS and 11.4% HbF. After an exchange procedure (2.825 ml) and the addition of 750 ml of blood the haemoglobin level was 11.8% g/dl, HbA had risen to 90.8%, HbS was reduced to 5.2% and HbF to 2.4% (Fig. 3). The exchange transfusion was performed without any side-effects and the operation and postoperative course were uncomplicated. Thirty days later, at follow-up, the HbA had returned to 85.9%, HbF was 4.0% and HbAs was 8.1%.

Comment

Although a similar reduction could have been obtained by single-unit exchanges, the ease and convenience for patient and attending physician using continuous-flow procedures represents a substantial advance in managing patients with these problems.

Although not common, homozygous Hbs production results in clinical syndromes which may become symptomatic with precipitating causes such as hyperoxia, infection, dehydration and acidosis. The availability of a technique which can rapidly and efficiently exchange HbA for Hbs with each crisis may be a life-saving measure.

Case 5

In May 1975 a 17-year-old girl presented with a 1 month history of backache and headaches. The headaches involved the whole cranium, but did not interfere with sleep, and the backache was associated with strenuous exercise involving the left lower lumbar region. Examination showed pallor of the mucous membranes and tenderness over the left lumbar area. Lymphadenopathy and splenomegaly were present. The peripheral blood smear showed a haemoglobin level of 6.3 g/dl, a white blood cell count of 26.3 x 10⁷/l with 97% lymphoblasts, and a platelet count of 68 x 10⁹/l. Examination of the bone marrow was diagnostic of acute lymphoblastic leukaemia (FAB-L1).

Induction was commenced with vincristine (2 mg) and prednisone (60 mg/d) given once a week. After 6 weeks of this therapy, bone marrow examination confirmed complete remission. Craniospinal prophylaxis was commenced with biweekly lumbar punctures for 3 weeks and thereafter monthly, with the administration of intrathecal methotrexate (12 mg/m²) alternating with cytosine arabinoside (30 mg/m²).

Maintenance therapy was commenced with thioguanine (40 mg/d), the dose being monitored by the peripheral blood count. During this period the patient developed pancytopenia with nausea and vomiting, requiring discontinuation of therapy. Four months after the induction period the peripheral blood count showed 21% lymphoblasts. Various combinations of drugs were then administered without success and 3 months later the blast count was 72% and the bone marrow had been totally replaced by leukaemic infiltrate. Periods of pancytopenia associated with infection required antibiotic therapy and granulocytic support. A decision was made to undertake bone marrow transplantation for drug-resistant disease. Although the donor and recipient were HLA-identical and MLC-non-reactive, the recipient's blood group was B Rh-positive, and that of the donor AB Rh-positive. Prior to the transplantation a preliminary plasmapheresis was performed against AB plasma. Six litres were exchanged over a period of 6 hours. The baseline anti-A titre before the procedure was 1:512 at 4°C and 22°C, on completion the anti-A titre was 1:16 at 4°C and 22°C. (Fig. 4).
Continuous-flow plasma exchange is a practical method for reducing naturally occurring iso-agglutinin so that bone marrow transplantation may be carried out across the barrier of blood group incompatibility. This is likely to be an unusual indication but again illustrates the central role that these instruments have come to occupy in leukaemia and bone marrow transplantation services.

**Discussion**

There are a number of clinical situations where symptoms of ill-health arise from an abnormality in either plasma or red cells. Examples of the former are hyperviscosity in the patient with myeloma, antibody-mediated disease such as Goodpasture's syndrome and rheus iso-immunization, immune complex disorders including rheumatoid arthritis, systemic lupus erythematosus, and neureitis or the autoimmune disease exemplified by myasthenia gravis.

Special interest centres on thrombotic thrombocytopenic purpura. Initially, plasmapheresis was shown to be effective in controlling the disease but subsequently infusion of fresh frozen plasma appeared to be equally effective. More recently the controversy has been reopened, and at present continuous-flow exchange against fresh-frozen plasma would appear to be the safest course to follow. It has been shown that after bites from certain venomous snakes the poison is limited to the plasma compartment and that the patients would benefit from removal of this pool of venom. Poisoning, for example by paracetamol overdose, is also amenable to treatment in this way.

In a pilot study we have reported the use of this technology as one approach to the mobilization of xanthomatous hyperlipidaemia. The resolution of the skin changes was associated with decrease in angina pectoris and may therefore reflect parallel reduction in coronary arterial atheromatous plaques. A prospective controlled study is now to be started to establish the possible role of long-term plasma exchange in the treatment of selected heterozygous hyperlipidaemic patients. Similarly, for the red cells, the ability to remove pathological haemoglobins from the circulation, particularly in situations where they pose short-term problems, is of great benefit to the patient.

In each of these situations exchange procedures using the older approach in which single units are collected and replaced are perfectly feasible but cumbersome and time-consuming. The introduction of this newer method centred around continuous-flow blood fraction separators has greatly increased the number of conditions amenable to control by a variety of exchange techniques. To avoid abuse of a costly procedure and to accumulate scientific data as opposed to further proliferation of anecdotal experience, it is stressed that patients being treated in this way be restricted to a limited number of centres and be entered on a carefully designed protocol study. Attempts to bypass such a structured approach will delay accumulation of the data necessary to place in perspective the role that continuous-flow plasma or red cell exchange has to play in clinical practice.

Although these machines provide excellent safeguards for the patient, each procedure is potentially dangerous. It is therefore mandatory to have constantly present a highly trained and expert staff thoroughly familiar with all aspects of machine operation. In practice we have
Premature gonadal failure

J. V. VAN DER MERWE

Premature gonadal failure is a relatively rare cause of amenorrhoea, with an approximate incidence of 4.8%. Apart from intense psychological, sociological, and practical implications of this condition, health-threatening associations are also well known. The association with other endocrinopathies is well documented. A higher incidence of ischaemic heart disease and severe osteoporosis is also associated with premature ovarian failure. Patients with premature gonadal failure are often subjected to unnecessary and extensive examinations for infertility. Furthermore, not much is known as regards the aetiology of this condition. This study was undertaken to illuminate the diagnostic procedures important in the management of these patients, as well as the aetiology of the condition.

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Date received: 11 June 1980.

Patients and methods

Fifty patients with hypergonadotrophic amenorrhoea under the age of 40 years were studied. Obvious cases of intersex or gonadal dysgenesis were excluded.

Depending on whether normal sexual characteristics were present, these patients were divided arbitrarily into a primary and secondary gonadal failure group, a classification of which is seen in Table I.

<table>
<thead>
<tr>
<th>TABLE I. CLASSIFICATION OF PATIENTS WITH HYPERGONADOTROPHIC AMENORRHEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary gonadal failure group</td>
</tr>
<tr>
<td>Pure gonadal dysgenesis</td>
</tr>
<tr>
<td>Ovotesticular dysgenesis</td>
</tr>
<tr>
<td>45,XO dysgenesis without Turner stigimate</td>
</tr>
<tr>
<td>45,X/46,XX mosaicism with Turner stigmate</td>
</tr>
<tr>
<td>X-chromosome deletions</td>
</tr>
<tr>
<td>X-chromosome translocations</td>
</tr>
<tr>
<td>Bilateral absent adnexa</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Secondary gonadal failure group</td>
</tr>
<tr>
<td>Irradiational gonadal failure</td>
</tr>
<tr>
<td>Post-surgical gonadal failure</td>
</tr>
<tr>
<td>Cytostatic-associated failure</td>
</tr>
<tr>
<td>Auto-immune oophoritis</td>
</tr>
<tr>
<td>Temporary ovarian failure</td>
</tr>
<tr>
<td>Idiopathic premature menopause</td>
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<tr>
<td></td>
</tr>
</tbody>
</table>
Comparison of Filtration to Continuous-Flow Centrifugation for Plasma Exchange

Lucille Wood, Rodney Bond, and Peter Jacobs

The University of Cape Town Leukaemia Centre and The Department of Haematology, Groote Schuur Hospital, Observatory, Cape Town, Republic of South Africa

The Asahi Plasmaflo Hollow Nylon Fibre Filtration System (n = 13) was directly compared to the NCI-IBM 2990 Continuous-Flow Blood Fraction Separator (n = 10) for plasma exchange. The systems were equally efficient in achieving plasma separation. There were significant differences favouring filtration for clearance of fibrinogen (P < 0.05), and the fourth component of complement (P < 0.01). Greater loss of urea (P < 0.05) was found after plasma exchange, using the cell separator. The flow characteristics were markedly different. In a standardized 4-L plasma exchange, filtration took place at 35 ml/minute, with a procedure time of 109 ± 45 minutes in contrast to centrifugation at a plasma flow collection rate of 19 ml/minute, requiring 208 ± 17 minutes. This time advantage for the former procedure was offset by 195 minutes required to regenerate the hollow nylon fibre unit and a further 90 minutes required for cleaning under strictly controlled aseptic techniques prior to reuse. Each filter was regenerated at least twice and reused without infection, but there was incremental loss of filtration efficiency demonstrated by decreasing clearance of an intravascular marker dye. In two of the 13 procedures using the Plasmaflo system, serious reactions necessitated termination of the procedure; this did not occur using the cell separator. Restriction of the number of times that the filter unit could be regenerated without loss of efficiency, the prolonged time required for regeneration and cleaning, coupled with the need for artificial vascular access to meet high blood flow rates required, limit the usefulness of this technique for plasma exchange.

Key words: filtration, hollow nylon fibres, continuous-flow centrifugation

INTRODUCTION

The ease and relative safety with which large volumes of plasma can be exchanged is the result of technical advances, culminating in the development of blood fraction separators and filtration techniques. Increasing experience has shown that such procedures can favorably influence the clinical course in a number of diseases, some of which are immunologically mediated [1–11]. However, much of the experience is anecdotal, and controlled studies will be necessary to define those entities where plasma exchange is likely to have major benefit. Among the other applications currently being evaluated are conditions characterised by quantitative and qualitative changes in normal plasma constituents, such as antibodies [5] and cholesterol [12]. More recently, improvement in patients with thrombotic thrombocytopenic purpura has been attributed to restoration of normal plasma constituents using this technique [13–15].

The increasing therapeutic application of this procedure has inevitably resulted in its use by less-experienced operators, so that maintenance and improvement in safety is an important technical objective. At the same time, rising costs have focused attention on new developments to enhance filtration efficiency, shorten exchange times, and explore methods for improving cost-effectiveness by reusing expensive items such as the filters.

In a prospective study, filtration using the Asahi Plasmaflo Hollow Nylon Fibre Unit was compared to continuous-flow centrifugation on the NCI-IBM 2990 Blood Fraction Separator. The physical requirements of each procedure, including filtration efficiency and donor response, were determined and the effects of regenerating the filter units on subsequent performance established.

PATIENTS

Patients with myasthenia gravis or immunoproliferative disorders were serially studied. The protocol was approved by the university and hospital ethics committee, the patients were fully informed of all aspects of either procedure, and signed consent was obtained.

MATERIALS AND METHODS

Haematological and biochemical measurements were determined before and after each procedure using the

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Coulter Counter Model S-Plus (Hialeah, FL) [16] and the Multi-Channel Analyser (Technicon, NY) [17], respectively. Standard techniques were used for determination of plasma protein by electrophoresis [18], immunoglobulins [19], haptoglobin [20], prothrombin time, partial thromboplastin time and fibrinogen [21], plasma haemoglobin [20], and total haemolytic complement as well as C3 and C4 components [22]. The presence of residual formaldehyde in the filters was determined by the Sjörenson's formal titration using the interaction with amino acids described by Schiff [23].

Blood cultures were performed on all patients undergoing plasma exchange, using the regenerated filtration units.

Filtration efficiency of the hollow nylon fibre filter was assessed by serially measuring clearance of the intravenous marker, Evans' blue dye [24]. Determinations were based on optical density and carried out at fixed intervals during the course of eluate formation. Serial studies were carried out using filters and comparing the initial slope for dye clearance to similar curves following one, and then two, regeneration procedures.

**Asahi Plasmaflow Hollow Nylon Fibre Filter**

Thirteen procedures were carried out using this system. The filters are made from cellulose di-acetate fibres with an effective surface area for exchange of 0.65 m² and a maximum pore size of 0.2 μm. These are housed in a synthetic polymer sleeve and the entire unit gas sterilized.

Whole blood filtration rates in excess of 100 ml/minute are required for optimal filtration, defined as filtrate formation at approximately 30% of flow rate. Although it is certainly possible to achieve flows of 200 ml/minute, this was found to be associated with hypotension, whereas rates between 100 and 120 ml of whole blood/minute were tolerated without fluctuations in blood pressure. To sustain these flow rates creation of an arterio-venous fistula or placement of a Scribner shunt [25] was necessary.

The output line from the patient (Fig. 1) passes sequentially through a roller pump, a blood microaggregate filter with airlock warning device (Pressure Isolator, model RSA 4410, Surgiprod, South Africa), a pressure monitor (Tycos Gauge, 5090/03), and into the fibre unit. Plasma leaving the pores is collected at the opposite end of the filter. Whole blood passing through the hollow nylon fibres is collected at a second microaggregate filter, filled with the same airlock device and pressure monitor, and flow adjusted to maintain transmembrane pressures below 100 mm of mercury by adjustment of flow rate. The filtered plasma is replaced with exactly equal volumes of 2.5% albumin in Ringer's lactate solution which is pumped into the whole blood line proximal to the second filter by means of a roller pump. The reconstituted whole blood is returned to the patient and since the volume removed accurately matches that replaced, the entire procedure is isovolaemic.

Prior to initiating the exchange, the filter is primed using 1,000 ml of physiological saline containing 50 mg (1 mg = 100 international units) of heparin as anticoagulant; further heparin is given at a rate of 20 mg for each litre exchanged.

Filters are regenerated after completion of the procedure by removing residual plasma with an air-rinse technique. Tap water is passed through the filter at a rate of 500 ml/minute for 2 hours to clean the core of the hollow nylon fibres. The tap water was not sterile, although it is the subject of regular water testing as defined by the local authority [26]. The rinsed filter is stored in 3% formaldehyde solution for a minimum of 48 hours, or until required. Prior to second or subsequent use, the formalin is removed by allowing 10% of sterile physiological saline to flow into the fibres and out through the plasma collection port. No formalin could be detected in the eluate [23]. Fluid from the final rinse was cultured for bacteria and fungi, using standard methods [27,28].

**NCI-IBM 2990 Continuous-Flow Blood Fraction Separator**

Ten procedures were carried out using this separator with ACD-A as anticoagulant and percutaneous venous access as previously described [29]. This instrument has
a reusable polycarbonate bowl which is cleaned and gas-
sterilised between procedures. Centrifugal speeds were
1600 rpm and total whole blood flow rates were 40 ml/
minute with isovolaemic plasma exchange taking place at
20 ml/minute.

Preparation of Equipment

Both systems required approximately 30 minutes for
priming, but the shorter procedure time for the filtration
method was offset by the laborious nature of filter regen-
eration, which required 195 minutes and a further 90
minutes for cleaning under strictly controlled aseptic
technique prior to reuse.

Patient Monitoring

The physical characteristics of each procedure were
constantly monitored, and they included whole blood
flow, filtration rates, and transmembrane pressures. Pa-
tients were under continuous observation for changes in
vital signs or development of symptoms, with special
attention given to hypotensive, haemolytic, or febrile
reactions. Before and after the procedure full blood count,
biochemical profile, and haemostasis screening tests were
carried out.

Statistics

Statistical analyses were carried out using standard
methods [30,31].

RESULTS

Filtration Rates

Twenty-three plasma exchanges were performed, each
of 4 L. Both filtration (n = 13) and centrifugal separation
(n = 10) were carried out in a cell support unit and
patients were constantly supervised by one of the invest-
gigators. The filters were regenerated using a strict aseptic
technique.

The plasma collection rates were significantly faster
with the membrane filtration technique at 35 ml/minute,
resulting in a mean procedure time of 109 minutes (SD
± 45 minutes) in comparison to 19 ml/minute using the
separator, with a mean procedure time of 208 minutes
(SD ± 17 minutes).

Haematologic Data

During the course of filtration minor differences were
demonstrable in packed cell volume, total white cell
count, and platelet count between the beginning and the
end of the procedure (Table I). Similar changes were
demonstrable during the course of continuous-flow filtra-
tion (Table II). However, when the percentage change
for these measurements was compared between the two
procedures, statistical significance (P < 0.001) was
achieved only for the total white cell count, where greater
rise was demonstrable in patients being treated by contin-
uous-flow centrifugation; at no time did white cell count
rise outside normal limits. It is not known whether this
rise reflects mobilization of the marginated granulocyte
pool or release of cells from the bone marrow, possibly
in response to circulation in the reusable polycarbonate
bowl. Appreciating that cell counts are concentration
measurements, we paid meticulous attention to maintain-
ing balance between plasma removed and replaced in
both systems to ensure that changes measured were ab-
solute and did not merely reflect differences in fluid
balance during the course of the procedure.

Biochemical Changes

During the course of filtration and continuous-flow
separation, changes were demonstrable between the be-
ginning and the completion of the procedure (Tables I,II)
and statistical changes were also evident when the two
systems were compared (Table III).

Plasma collection was achieved with equal facility in
both systems. The filtration technique more efficiently
removed fibrinogen (P < 0.05) and the fourth compo-
nent of complement (P < 0.05). In contrast, centrifuga-
 tion resulted in greater reduction of urea (P < 0.05).

Filtration Efficiency

In 500 consecutive procedures, the separation charac-
teristics of the reusable polycarbonate bowl in the NCI-
IBM 2990 instrument were found to be completely stable
and predictable [Wood and Jacobs, unpublished].

The Asahi Plasmaplo Hollow Nylon Fibre Filters
showed an incremental loss of efficiency when the slope
of the dye elution curve was compared between first,
second, and third procedures (P < 0.01) (Fig. 2). In
addition, there was reduction in clearance of the gam-
maglobulin fraction with each regeneration and reuse of
a particular filter (P < 0.02).

Procedure Safety

There were no untoward reactions in the group of
patients (n = 10) who underwent plasma exchange using
the NCI-IBM continuous-flow blood fraction separator.

Two of the patients (n = 13) undergoing exchange
using the Asahi Plasmaplo Hollow Nylon Fibre Filtration
System required termination of the procedure because of
serious reaction. The first patient became hypotensive
early during the procedure and failed to respond to infu-
sion of intravenous fluids. This patient subsequently
underwent plasma exchange using the filtration system
on a further four occasions without incident. In a second
patient, haemolysis occurred associated with an elevation
of the free plasma haemoglobin after 3,500 ml of eluate
had been collected. The procedure was discontinued but
### TABLE I. Haematological and Biochemical Data for Asahi Plasmaflo Hollow Nylon Fibre Filtration System

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Preprocedure (Mean ± 1 SD)</th>
<th>Postprocedure (Mean ± 1 SD)</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed cell volume (%)</td>
<td>27 ± 7</td>
<td>26 ± 7</td>
<td>−4</td>
</tr>
<tr>
<td>Granulocyte count (× 10⁹/µl)</td>
<td>4.9 ± 2.8</td>
<td>6.4 ± 3.6</td>
<td>30</td>
</tr>
<tr>
<td>Platelet count (× 10⁹/µl)</td>
<td>124 ± 80</td>
<td>112 ± 64</td>
<td>−10</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.2 ± 1.1</td>
<td>0.61 ± 0.3</td>
<td>−81</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>1.2 ± 0.4</td>
<td>0.32 ± 0.3</td>
<td>−73</td>
</tr>
<tr>
<td>(g/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>76.5 ± 13.4</td>
<td>69.5 ± 12.1</td>
<td>−9</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>27.8 ± 18</td>
<td>25.8 ± 17</td>
<td>−7</td>
</tr>
<tr>
<td>Uric acid (mmol/l)</td>
<td>0.27 ± 0.07</td>
<td>0.23 ± 0.06</td>
<td>−15</td>
</tr>
<tr>
<td>Lactic dehydrogenase (units/l)</td>
<td>283 ± 41</td>
<td>195 ± 58</td>
<td>−32</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>57.0 ± 4.4</td>
<td>53.7 ± 8.3</td>
<td>−6</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>34.6 ± 2.4</td>
<td>38.0 ± 4.5</td>
<td>+10</td>
</tr>
<tr>
<td>Total globulin (g/l)</td>
<td>6.5 ± 3.0</td>
<td>5.6 ± 1.4</td>
<td>−14</td>
</tr>
<tr>
<td>IgG (g/l)</td>
<td>3.8 ± 2.4</td>
<td>2.9 ± 1.7</td>
<td>−24</td>
</tr>
<tr>
<td>IgA (g/l)</td>
<td>1.0 ± 0.9</td>
<td>0.4 ± 0.38</td>
<td>−60</td>
</tr>
<tr>
<td>IgM (g/l)</td>
<td>0.6 ± 0.4</td>
<td>0.4 ± 0.26</td>
<td>−33</td>
</tr>
<tr>
<td>Total complement (units/ml)</td>
<td>232 ± 53</td>
<td>108 ± 48</td>
<td>−53</td>
</tr>
<tr>
<td>C3 (mg/dl)</td>
<td>91 ± 28</td>
<td>55 ± 17</td>
<td>−39</td>
</tr>
<tr>
<td>C4 (mg/dl)</td>
<td>17 ± 6</td>
<td>11 ± 4</td>
<td>−35</td>
</tr>
</tbody>
</table>

Percentage change is calculated by the difference between the mean levels before and after completion of the procedure expressed as a percentage of the starting value.

the same filter regenerated successfully and the patient underwent further plasma exchanges without incident.

Culture for bacteria and fungi in the patients and the regenerated filters failed to reveal any abnormality.

Intravenous supplementation of calcium and potassium were administered prophylactically to both groups of patients, so that comments about changes in these cations is not possible. Donor symptomatology and vital signs of all patients in both groups were no different, apart from the two incidents described above.

**Cost**

A single filtration procedure cost $672.00 and that on the cell separator $385.00 (Table IV).

**DISCUSSION**

Plasma exchange using continuous-flow blood fraction separators is now an established technique for the management of patients with plasma hyperviscosity, although it may be useful in the treatment of a number of diseases mediated by circulating immune complexes or antibodies.

![Fig. 2. Changes in efficiency following hollow nylon fibre filter regeneration. The intravascular marker dye, T-1824 or Evans blue, is measured at 620 nm against the eluate in ml. There is decreasing efficiency between first use of the filter (●, n = 3), second use (△, n = 2), and third use (■, n = 4).](image-url)
Plasmapheresis Compared to Filtration

TABLE II. Haematological and Biochemical Data NCI-IBM 2990 Cell Separator

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Preprocedure (Mean ± 1 SD)</th>
<th>Postprocedure (Mean ± 1 SD)</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed cell volume (%)</td>
<td>37 ± 1</td>
<td>36 ± 2</td>
<td>-2</td>
</tr>
<tr>
<td>Granulocyte count (× 10⁹/l)</td>
<td>5.2 ± 1.2</td>
<td>8.9 ± 2.1</td>
<td>+69</td>
</tr>
<tr>
<td>Platelet count (× 10⁹/l)</td>
<td>228 ± 62</td>
<td>210 ± 37</td>
<td>-8</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.8 ± 0.7</td>
<td>2.2 ± 0.9</td>
<td>-43</td>
</tr>
<tr>
<td>Haptoglobin (g/l)</td>
<td>2.0 ± 0.5</td>
<td>0.7 ± 0.3</td>
<td>-64</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>76.5 ± 13.4</td>
<td>72.7 ± 5.8</td>
<td>-5</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>3.0 ± 0.5</td>
<td>2.0 ± 0.3</td>
<td>-33</td>
</tr>
<tr>
<td>Urate (mmol/l)</td>
<td>0.21 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>-19</td>
</tr>
<tr>
<td>Lactic dehydrogenase (units/ml)</td>
<td>357 ± 38</td>
<td>205 ± 59</td>
<td>-42</td>
</tr>
<tr>
<td>Total protein (g/l)</td>
<td>58.0 ± 6.0</td>
<td>48.0 ± 5.0</td>
<td>-17</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>32.3 ± 3.2</td>
<td>33.2 ± 6.1</td>
<td>+3</td>
</tr>
<tr>
<td>Total globulin (g/l)</td>
<td>7.7 ± 1.6</td>
<td>5.0 ± 1.2</td>
<td>-35</td>
</tr>
<tr>
<td>IgG (g/l)</td>
<td>5.9 ± 1.2</td>
<td>3.9 ± 0.9</td>
<td>-34</td>
</tr>
<tr>
<td>IgA (g/l)</td>
<td>2.0 ± 0.38</td>
<td>0.78 ± 0.18</td>
<td>-61</td>
</tr>
<tr>
<td>IgM (g/l)</td>
<td>0.78 ± 0.23</td>
<td>0.4 ± 0.15</td>
<td>-49</td>
</tr>
<tr>
<td>Total complement (units/ml)</td>
<td>204 ± 54</td>
<td>111 ± 25</td>
<td>-54</td>
</tr>
<tr>
<td>C3 (mg/dl)</td>
<td>101 ± 24</td>
<td>53 ± 10</td>
<td>-48</td>
</tr>
<tr>
<td>C4 (mg/dl)</td>
<td>12 ± 3</td>
<td>11 ± 2</td>
<td>-8</td>
</tr>
</tbody>
</table>

Percentage change is calculated by the difference between the mean levels before and after completion of the procedure expressed as a percentage of the starting value.

[2, 28, 32]. Nevertheless, there is not yet consensus about all the indications for plasmapheresis and therefore, not surprisingly, a very wide range of clinical syndromes are currently being intensively studied. These investigations have been encouraged by the extensive testing and relative safety of these separators [2], although it is not yet adequately appreciated that morbidity and even mortality is associated with their use [33]. Furthermore, apart from the capital outlay for the purchase of the instruments and the salaries for the staff, the cost of plastic and exchange fluid is currently around $350.00 for each procedure.

It is against this background that new techniques must be assessed. Membrane filtration, using the hollow nylon fibre filter, has been demonstrated as an effective means for plasma exchange [34–36], achieving 60% reduction of total protein from initial levels and a decrease of fibrinogen by 50% in the course of a single procedure; our results compare favourably with these figures. Packed cell volume changes, although small, were significant for the individual procedures (P < 0.01) during the course of the plasma exchange but were not different for the two methods (P < 0.05). The significantly elevated total white cell count is different between the two procedures and contrary to published reports [34–36]. The increase was never outside the upper limit of normal, and redistribution of the margined granulocyte pool would appear the most obvious explanation for elevation of count in the absence of infection or the administration of drugs known to produce similar change. It is not known whether this may reflect elution of some stimulator substance from the reusable bowl or the membrane, since a similar change occurred with the filters.

Although the mean packed cell volume and platelet counts were lower in a group of patients treated on the filters than those on the separators there were, within each series, subjects who could be studied at roughly comparable levels. In each of these individual comparisons the data were consistent with that recorded for the total group. Similarly, it is apparent that mean blood urea levels, although not those for creatinine, were also different between the two groups. The explanation for this is found in one patient with severe renal failure altering the mean level. Here again there were sufficient observations lying in the normal range amongst procedures carried out with the filtration technique to ensure that the group characteristics were reliable and reflected the same pattern of change seen before and after individual plasma exchanges. The results of statistical analysis when com-
TABLE III. Haematological and Biochemical Data

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Filtration</th>
<th>Centrifugation</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed cell volume</td>
<td>-4</td>
<td>-2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Granulocyte count</td>
<td>+30</td>
<td>+70</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelet count</td>
<td>-10</td>
<td>-8</td>
<td>N.S.</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-80</td>
<td>-44</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>-77</td>
<td>-62</td>
<td>N.S.</td>
</tr>
<tr>
<td>Creatinine</td>
<td>-18</td>
<td>-11</td>
<td>N.S.</td>
</tr>
<tr>
<td>Urea</td>
<td>-13</td>
<td>-27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urate</td>
<td>-18</td>
<td>-20</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>-36</td>
<td>-42</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total protein</td>
<td>-6</td>
<td>-10</td>
<td>N.S.</td>
</tr>
<tr>
<td>Albumin</td>
<td>-3</td>
<td>-4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total globulin</td>
<td>-32</td>
<td>-33</td>
<td>N.S.</td>
</tr>
<tr>
<td>IgG</td>
<td>-24</td>
<td>-34</td>
<td>N.S.</td>
</tr>
<tr>
<td>IgA</td>
<td>-60</td>
<td>-62</td>
<td>N.S.</td>
</tr>
<tr>
<td>IgM</td>
<td>-33</td>
<td>-46</td>
<td>N.S.</td>
</tr>
<tr>
<td>Total complement</td>
<td>-47</td>
<td>-43</td>
<td>N.S.</td>
</tr>
<tr>
<td>C3</td>
<td>-32</td>
<td>-49</td>
<td>N.S.</td>
</tr>
<tr>
<td>C4</td>
<td>-30</td>
<td>-12</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Comparison is made between percentage change during procedures carried out on the Asahi Plasmaflo Hollow Nylon Fibre Filtration System and the NCI-IBM 2990 Cell Separator. The difference between percentage change achieved during the course of the two procedures was then compared and statistical significance achieved only for granulocyte count, fibrinogen, urea, and C4.

TABLE IV. Comparison of Costs

<table>
<thead>
<tr>
<th>Item</th>
<th>Filtration ($)</th>
<th>IBM ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubing and filters</td>
<td>310</td>
<td>77</td>
</tr>
<tr>
<td>Exchange fluid</td>
<td>240</td>
<td>240</td>
</tr>
<tr>
<td>Flushing saline</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Priming fluid and filters</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>ACD-A</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>Heparin</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Time costs</td>
<td>80</td>
<td>40</td>
</tr>
<tr>
<td>Totals</td>
<td>672</td>
<td>385</td>
</tr>
</tbody>
</table>

The figures are those currently prevailing in the Cell Support Unit at the University of Cape Town and Groote Schuur Hospital during the study period in 1982.

Comparing filtration to centrifugation are not influenced by the different mean urea levels. Unlike the plasma fibrinogen level which fell with both procedures, significantly more so with the filtration technique, only minor changes were recorded in the prothrombin and partial thromboplastin times: None of these reached statistically significant levels.

The advantages of the Plasmaflo Hollow Nylon Fibre Filter System include faster filtration rates, shorter procedure times, and a cell-free eluate obtained with ease of operation and patient accessibility. The first two of these considerations are at the expense of high whole-blood flow rates required by this technique, so that artificial venous access in the form of an arterio-venous shunt is a necessity. It needs to be emphasized that, although no difficulties were experienced in this study, the creation of such vascular access is not without risk, including disconnection of the exteriorised shunt with exsanguinating haemorrhage or shunt occlusion. However, had percutaneous venous cannulation been used, the filtration rate with the Plasmaflo system would be less than the corresponding rate for the centrifugal continuous-flow method, with both a time penalty and an inability to sustain flow rates essential for efficient plasma separation. The hardware necessary for both methods is equally mobile so that there is no advantage for the Plasmaflo system in treating patients nursed away from the main separator unit, as would occur in the intensive care wards.

Excluding the initial cost of the two systems and staff salaries, the disposable plastics for each procedure were found to be $310.00 for the cellulose acetate hollow nylon fibre filter compared to $77.00 for the cell separator. To be equally cost-effective, each filter would need regeneration. This requirement, although time consuming and cumbersome, can be achieved, and the use of regenerated filters was associated with a surprising lack of side-effects. The fall-off of efficiency, however, precludes a sufficient number of regeneration cycles necessary to compete with the cost of disposables employed in operating the separator. In addition, the potential risk to the patient, although not realised in our studies, together with the time required by a skilled member of staff, are compelling arguments against filter regeneration. Continued experience with this system and the development of expertise by staff members may, theoretically at least, partially offset these disadvantages.
Two serious reactions were encountered with the use of the Plasmaflo Hollow Nylon Fibre Filter System. The first was considered an allergic reaction to antigen contamination in the plasma protein replacement solution. The second was caused by haemolysis during the procedure. The transmembrane pressure did not exceed 100 mm of mercury and was insufficient to cause red cell breakdown. This patient suffered from Bence-Jones myeloma and it is theoretically possible that protein coating the membrane may have been responsible for this reaction. It is noteworthy that subsequent regeneration and reuse of the filter was uneventful.

It is recognised that plasma exchange separation is a function of centrifugal speed, packed cell volume, and operator selection of plasma and red cell interface. Furthermore, there is a need to adjust blood flow and the addition of anticoagulant to avoid the development of symptoms due to hypocalcaemia when ACD-A is employed. In contrast the membrane filtration system has, as its major limitation, the need to maintain whole blood flow rates at or above 100 ml/minute in order to obtain optimum efficiency. These factors which influence plasma collection are fundamentally different between the two techniques, but this prospective study showed that the Asahi Plasmaflo Hollow Nylon Fibre Filtration System has equal clinical utility to the NCI/IBM 2990 Continuous-Flow Blood Fraction Separator when comparable volumes of plasma are exchanged. However the inferior cost-effectiveness of the filtration system together with the need for the creation of artificial vascular access are considered to limit its clinical usefulness at the present time.

ACKNOWLEDGMENTS

Supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the Medical Research Council, and the National Cancer Association. We thank Abbotts for donation of the Asahi filtration units, Jackie Davies and Dorothy Banner for typing, Jeanne Walker for medical illustration, Sheila Katcher for bibliographic assistance and the medical superintendent, Dr. H.R. Sanders, of Groote Schuur Hospital, for permission to publish.

REFERENCES


Studies on Platelets Contained in Eluates Following Filtration Leukapheresis

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From the Leukaemia Centre, University of Cape Town and the Departments of Haematology and Medical Physics, Groote Schuur Hospital, Cape Town, South Africa

Platelets eluted from nylon fiber filters after filtration leukapheresis have been studied. The platelet yield from 61 routine donations was $1.25 \pm 0.18 \times 10^{11}$ (mean ± SEM) corresponding to $1.78 \times 10^{9}$ per 500 ml blood processed. Filtered platelets labeled with radiochromate demonstrated reduced recovery in vivo 15 minutes after infusion (38.5 ± 1.7%) when compared to the control value (68.5 ± 6.8%, p < 0.001). The survival of those platelets remaining in the circulation after 15 minutes did not however differ from the control value. ADP (10 μM, 100 μM, 1 mM), adrenaline (100 μM) and collagen (7.25 mg/ml) added in vitro induced less aggregation of filtered platelets than normal control platelets and electron microscopy revealed structural abnormalities. It is concluded that recipients of granulocyte transfusions obtained by filtration leukapheresis are unlikely to be benefited by the platelets contained in these transfusions.

The high mortality from infection and hemorrhage that is associated with bone marrow suppression, has led to the development of improved methods of collecting granulocytes and platelets for the supportive care of patients in this clinical situation. These methods employ machines which selectively remove granulocytes and platelets from donor blood circulated through them, either by continuous or discontinuous centrifugation or by filtration through a nylon fiber filter. 13 Whereas both methods are used for harvesting granulocytes, only centrifugation is used as a method of collecting platelets. It has been observed that platelets are eluted together with granulocytes obtained by filtration leukapheresis. 9,12 The platelets might be of benefit to patients receiving granulocytes obtained by this method, as has been reported in patients receiving platelets obtained by other techniques. 4,6 Such benefit would be contingent upon the provision of platelets in sufficient number and of proper function. Since no such data was previously available, we have undertaken a study of these platelets and the results obtained form the basis of this communication.

Materials and Methods

Our procedure for routine filtration leukapheresis is to use two Leuco-Pak 14 nylon fiber filters set in parallel through which heparinized donor blood is perfused for 90 minutes at a flow rate of 40 ml per minute. Thereafter the adherent cells are eluted with 1,000 ml per filter of an ACD (125 ml), plasma (125 ml) and saline (750 ml) mixture of pH 6.5, accompanied by gentle mechanical tapping of the filters. The total number of platelets contained in each eluate was calculated as the product of the volume of the eluate and the platelet concentration. Platelet counts were performed on a Coulter Counter Model FN and data were obtained on 61 leukaphereses.

Platelet survival was studied in seven members of the hospital medical staff. Four-hundred ml of blood was collected into 60 ml ACD (NIH solution-A) and centrifuged at $300 \times g$ for 15 minutes in order to obtain platelet-rich plasma (PRP). The PRP was transferred to a satellite bag and the red blood cells returned to the donor. The pH of the PRP was adjusted to 6.3 ± 0.2 by the addition of approximately 15 ml of ACD and spun at 1,500 $\times g$ for 15 minutes in order to prepare a platelet pellet. All but 20 ml of the supernatant platelet-poor plasma (PPP) was transferred to a satellite bag. The platelet pellet was gently resuspended in the remaining 20 ml PPP and incubated with 400 μCl of isotonic sodium radio-
FILTERED PLATELETS

chromate ($\text{Na}_2\text{HCrO}_4$) at room temperature for 30 minutes. After incubation the platelets were washed twice with approximately 100 ml of PPP, particular care being taken to exclude contaminating red blood cells from the final labelled platelet suspension. Nonplatelet bound radioactivity constituted less than two per cent of the radioactivity in the preparation for injection. After injecting the labeled platelets, 5 ml samples of whole blood were obtained from the opposite arm at 15 minutes, one hour, three hours and daily thereafter whenever possible for eight days by which time the radioactivity had fallen to approximately 15 per cent of the 15 minute value. The whole blood samples were counted in a gamma spectrometer to a standard deviation of less than two per cent.

Platelet recovery is defined as the percentage of platelet-bound radioactivity (PBR) remaining in the blood 15 minutes after infusion. This value was calculated from the formula:

$$\frac{\text{PBR/ml Blood} \times \text{Blood Volume}}{\text{Total PBR injected}} \times 100$$

The blood volume was estimated from height and weight measurements.\textsuperscript{16}

In view of the uncertainty concerning the most appropriate model for analysing platelet survival curves,\textsuperscript{16} the observed data were fitted to linear and exponential functions, using a UNIVAC model 1106 computer, and platelet mean lifespan (MLS) was calculated for each decay model. For linear functions, the values of radioactivity $y_t$ at time $t$, were fitted by the method of linear least squares to the function $y(t) = a \cdot e^{bt}$ from which platelet MLS was obtained as MLS = $a/b$.

For exponential function $y(t) = a \cdot e^{bt}$ the data were fitted to the equivalent linear relationship, $\ln y(t) = \ln a - bt$ by the linear least squares method. Goodness-of-fit for linear and exponential

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Fig. 1. Preparation of labeled filtered platelets. Blood was sampled distal to the filter and platelet radioactivity (○) and total platelet count (□) recorded as a percentage of the prefiltration level. Values expressed as the mean ± SEM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Fig. 2. Linear plot of platelet survival data for control (○) and filtered (●) platelets, and the least-squares fit to the data. Values expressed as the mean ± SEM. Platelet MLS by a linear function is the point of intersection of the graph with the x axis: 213 hours for the control and 203 hours for the filtered platelets.}
\end{figure}
Table 1. Platelet Mean Lifespan (MLS) (Mean ± SEM) for Control and Filtered Platelets Calculated by Linear and Exponential Functions, and an Evaluation of Goodness-of-fit by the Chi-Square Test

<table>
<thead>
<tr>
<th>Platelets MLS (hour)</th>
<th>Goodness-of-fit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linear</td>
</tr>
<tr>
<td>Control</td>
<td>213 ± 15</td>
</tr>
<tr>
<td>Filter</td>
<td>203 ± 8</td>
</tr>
</tbody>
</table>

models was assessed by the chi-square test. The seven individual results for platelet recovery and platelet MLS were means to give a single value for the group.

Three to five months after the completion of the control measurement, the survival of filtered platelets was studied in the same seven volunteers, thereby enabling each individual to act as his own control. The most appropriate experimental design would have been to label platelets after their elution from the filter following a routine donation. However, because these eluted platelets could not be separated adequately from contaminating red blood cells prior to labeling, (essential since erythrocytes have a much higher affinity for radiocromate than do platelets), and because of uncertainties about the labeling characteristics in the eluting fluid medium, an alternative method of obtaining filtered platelets was devised, comparable, as far as was possible with the routine donation procedure. Blood was collected into ACD and platelets were labeled with radiocromate as for the control study. The red blood cells were returned to the donors and then 350 to 400 ml of blood were collected into 2,500 units of heparin, (two blood collections were necessary, since ACD blood was needed for labeling and heparinized blood is needed for filtration-elution). The labelled platelet preparation (20 ml) was added to this heparinized blood. The blood and added platelets were mixed by inversion and then by means of a rotary pump continuously circulated at a rate of 20 ml/minute through a single Leuco-Pak filter for 90 minutes. During this time one ml aliquots were sampled distal to the filter to observe the efficiency with which the filter extracted the platelets, and to ascertain whether the prior radioactive labeling of the platelets had in any way modified their behavior in relation to the filtration process. On completion of the procedure, the filter was removed and the adherent platelets eluted as described above. The eluate was concentrated by centrifugation and an aliquot was removed for platelet aggregometry. The remainder of the eluate measuring approximately 35 ml and containing a mean of 6.6 x 10^10 platelets, was injected

Table 2. Maximum Aggregation of Platelets Contained in Seven Specially Prepared Eluates, in Response to Collagen, Adrenaline and ADP

<table>
<thead>
<tr>
<th>Case</th>
<th>Collagen 7.25 mg/ml</th>
<th>Adrenaline 100 μM</th>
<th>ADP 100 μM</th>
<th>ADP 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>F</td>
<td>C</td>
<td>F</td>
</tr>
<tr>
<td>1</td>
<td>86</td>
<td>18</td>
<td>89</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>74</td>
<td>20</td>
<td>81</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>8</td>
<td>70</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>5</td>
<td>79</td>
<td>33</td>
<td>80</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>23</td>
<td>73</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>91</td>
<td>10</td>
<td>84</td>
<td>40</td>
</tr>
</tbody>
</table>

Mean | 71† | 18† | 82† | 36† | 75 | 43 | 75 | 48 |
F/C (%) | 25 | 44 | 57 | 64 |

Concentrations quoted are final concentrations, and the per cent aggregation that observed at eight minutes.

C = Control platelets; F = Filtered platelets.

* Not performed.
† Excludes case 4 where control platelets failed to aggregate.
into the volunteers. Subsequent sampling and calculation of platelet recovery and platelet MLS being the same as for the control study.

Platelet aggregometry was performed on aliquots of the seven eluates as prepared above, and the eluates obtained from ten donors following routine leukapheresis for patients requiring supportive care. Citrated blood was also obtained from these persons in order to prepare control platelets and also to prepare PPP. The eluate was centrifuged for 5 minutes at 100 × g. The upper platelet-containing layer was removed and then the platelets were concentrated by centrifuging at 1100 × g for 10 minutes. The platelets were then resuspended in control PPP in order to restore the pH, fibrinogen and calcium ions necessary for aggregation. Both control and filtered platelets were adjusted to a concentration of 300 × 10^9/l. Aggregometry was performed within two hours using a Payton twin-channel aggregometer. The aggregating agents were used in the following final concentrations: ADP 10 μM, 100 μM, 1 mM, adrenaline 100 μM and collagen 7.25 mg/ml. The effect of these agents was recorded as the maximal increase in light transmission after 8 minutes on a scale where PRP corresponded to 0% transmission and PPP to 100%. The individual results obtained for control and filtered platelets with each agent were meaned and the significance of the difference between means assessed by Students t test.

Electron microscopy was performed on platelets from three eluates obtained for therapeutic purposes. Citrated blood for control platelets was obtained from the same three donors. The control PRP and the eluates were first fixed in phosphate buffered gluteraldehyde and post fixed in osmium tetroxide. The samples were dehydrated in 2% uranyl acetate in increasing concentrations of acetone, embedded, sectioned and stained with saturated uranyl acetate and 0.2% lead citrate.

Results

The average platelet yield per run was 1.25 × 10^10, (n = 61, SEM = 0.18 × 10^10), corresponding to 1.78 × 10^10 per 500 ml blood processed. The total platelet count and PBR in samples of blood having passed through the filter is demonstrated in Figure 1. Compared with the prefiltration values, a fall in both PBR and total platelet count was observed. The labeled platelets were initially extracted less well than the unlabeled platelets, the former falling to 18 per cent and the latter to 9 per cent of their prefiltration level at 1 minute. This difference was not however significant at the 0.01 level and the labeling procedure per se appears not to have adversely affected the platelets in respect to the filtration process.

The values for the total platelet count and PBR after 90 minutes of filtration were 26 and 30 per cent respectively. These values were greater than the corresponding 1 minute values, suggesting a net loss of platelets from the filter during filtration. The differences were not significant (0.01 < p < 0.05), and the majority of platelets can be considered to have been retained within the filter throughout the period of filtration.

Recovery 15 minutes after injection of control platelets was 68.5 per cent which is in good agreement with other series.13-15 The recovery of 38.5 per cent ± 1.7 of the filtered labeled platelets was significantly less than the control value (p < 0.001). This low recovery is most likely to be the consequence of damage caused to the platelets during filtration and/or elution, resulting in their rapid removal from the circulation. Lesser degrees of damage permitting the

| Table 3. Maximum Aggregation of Platelets Contained in Ten Eluates Obtained by Routine Filtration Leukapheresis, in Response to Collagen, Adrenaline and ADP |
|-----------------|----------|----------|----------|----------|----------|----------|----------|----------|
| Collagen 7.25 mg/ml | Adrenaline 100 μM | ADP 10 μM | ADP 100 μM |
| C | F | C | F | C | F | C | F |
| Mean | 62 | 11 | 78 | 16 | 74 | 21 | 77 | 43 |
| SEM | 6 | 4 | 3 | 2 | 2 | 6 | 3 | 6 |
| F/C (%) | 16 | 20 | | | | | | |
| p | <0.001 | <0.001 | | | | | | |

Concentrations are final concentrations, and per cent aggregation that observed at eight minutes. C = Control platelets; F = Filtered platelets.
return of some platelets to the circulation presumably accounts for the slight increase in recovery observed during the first three hours of the survival study (Fig. 2).

The control platelet MLS was 213 ± 15 hours (mean ± SEM) and 116 ± 8 hours by the linear and exponential models respectively. These results are in agreement with other series.\textsuperscript{5,6,14} Filtered platelet MLS by linear and exponential functions was 203 ± 8 hours and 118 ± 5 hours respectively (Table 1). These values were not significantly different from the control. Although approximately half the infused filtered platelets are irreversibly damaged, as demonstrated by the low mean recovery value, those platelets not initially removed from the circulation survive normally. Whereas the control survival data are satisfactorily described by both linear and exponential functions, only the linear function fits the filtered data, the exponential fit being rejected. Accordingly only the linear least squares fit is presented (Fig. 2).

Maximum aggregation after eight minutes in response to the aggregating agents used, was in all instances less for the filtered platelets than for the control platelets. Using concentrations of adrenaline (100 µM) and ADP (100 µM, 1 mM) greater than is generally used for routine testing, did not significantly increase the degree of aggregation. Thus for all aggregating agents the difference between filtered and control platelets was significant (p < 0.01 or p < 0.001).

The data obtained on the seven specially prepared eluates are presented in Table 2, and the results obtained on the ten routine eluates are presented in Table 3.

Abnormalities of ultrastructure were noted in all three eluates studied and comprised degranulation or complete disruption of cells, (Fig. 3). Control platelets were not altered structurally.

Discussion

Continuous and discontinuous centrifugation permits the selective harvesting of granulocytes or platelets according to patient requirement, whereas nylon fiber filtration is employed for granulocyte harvesting alone.\textsuperscript{3} MacPherson et al.\textsuperscript{9} documented an average yield of 0.64 × 10\textsuperscript{11} platelets from five filtration leukaphereses, and Russel and Powles\textsuperscript{10} also noted the presence of platelets in eluates, but no data were presented. We have documented an average of 1.25 × 10\textsuperscript{11} platelets per leukapheresis. This amount bears favorable numerical comparison with 1.03 × 10\textsuperscript{11} for double manual plateletapheresis but not with mechanical methods for example 2.5 – 3 × 10\textsuperscript{11} for six cycle discontinuous centrifugation in ACD.\textsuperscript{4}
The function of the filtered platelets was greatly impaired. Deficient aggregation in response to three physiologic agents was observed. Although no ultrastructural abnormalities to account for abnormal aggregation were observed following discontinuous centrifugation, it is the present study defective aggregation can, at least in part, be explained by platelets having already undergone partial degranulation. Additional evidence of platelet damage was provided by the 51Cr study in which approximately one half of the platelets were removed from the circulation within 15 minutes of infusion. Those platelets remaining in the circulation, even though they have a normal lifespan, are unlikely to be of value since their number (approximately $0.6 \times 10^{11}$) is less than that which could be expected to obtain satisfactory increments in recipients. Any effect is likely to be further diminished, by the presence of the associated fever and infection for which purpose the granulocyte transfusions are being administered.

Acknowledgments
The authors are indebted to Dr. A. Ferrant of the Department of Haematology, University of Louvain, Belgium for many helpful discussions. The technical assistance of Mr. B. Kossew, Ms. G. Randall and Ms. J. Hughes, is gratefully acknowledged.

References
Platelet Collection Using the IBM 2997 Cell Separator

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The University of Cape Town Leukaemia Centre and The Department of Haematology, Groote Schuur Hospital, Observatory, Cape, Republic of South Africa

Platelets were collected using the dual-channel module on the IBM 2997 Blood Fraction Separator. We carried out 320 procedures to harvest platelets for therapeutic purposes and yielded 5.1 ± 1.5 × 10¹¹ platelets (mean ± SD). Infusion into previously unsensitized recipients with hypoplastic or aplastic thrombocytopenia achieved increments at 1 hr of 19 ± 7.3 × 10⁹/liter/m² (mean ± SD) and at 24 hr of 15 ± 6.3 × 10⁹/liter/m². The only consistent donor reaction was mild hypocalcaemia, easily corrected by calcium gluconate infusion. Changes in donor packed-cell volume and white cell count were not statistically altered (p > 0.05) but donor platelet counts fell from 216 ± 43.1 × 10⁹/liter to 162.5 ± 41.7 × 10⁹/liter (mean ± SD; p < 0.01). Additional plateleterapheresis were carried out in seven normal volunteers, using the same technique, in order that the function of the harvested platelets could be studied. Following radiochromium labelling and reinfusion into the same donors, normal in vivo recoveries were obtained at 10 min (59.4 ± 3.4%; mean ± SD) and platelet mean life span was also normal (218 ± 12 hr; mean ± SD). Furthermore, in vitro platelet factor III availability and aggregation patterns of the harvested platelets did not differ from control values and their ultrastructural appearance was normal.

Key words: survival, aggregometry, ultrastructure, therapeutic transfusions

INTRODUCTION

Thrombocytopenic bleeding, due to bone marrow suppression, exposes patients to significant morbidity and mortality. Clinical studies have demonstrated the value of platelet transfusions in the management of such individuals [1]. The effectiveness of this therapy, however, depends on obtaining a sufficient number of normally functioning platelets from donors for infusion, a problem that has largely been resolved by the use of blood cell separators [2–4]. Cell collection, including platelets, has been based on adaptations of plasmapheresis techniques [5]. In the earlier instruments, centrifugation took place in reusable polycarbonate bowls, but disposable plastic channels have now replaced these cumbersome and potentially dangerous components of the older instruments [6–8].

More recently, the collection channel has been redesigned to enhance the efficiency of platelet collection further [9], and we report our experience of plateleterapheresis with this dual-channel module, used in conjunction with the IBM 2997 continuous-flow blood cell separator.

MATERIALS AND METHODS

Platelet Donations for Therapeutic Purposes (N = 320)

Donors fulfilling standard criteria [10] were given a full description of the nature of the procedure and gave written informed consent. Full blood counts [11], differential [12], and platelet counts [11,13], were performed on each donor before and after plateleterapheresis. This procedure was carried out using the dual-channel module on the IBM 2997 Cell Separator following priming with physiological saline containing acid-citrate-dextrose (ACD-A; Fenwal Laboratories, Morton Grove, IL) at a ratio between 1.65 and 1.75 to whole blood as determined by donor packed cell volume. Blood was removed and returned to the antecubital veins, using a 16 gauge cannula (Intracath, Johnson & Johnson, South Africa).

The flow rate was 42 ± 8 ml/min at a centrifuge speed of 2400 rpm. Anticoagulation was maintained during the procedure with one part of acid-citrate-dextrose to seven parts of whole blood. Blood samples were collected during the procedure in order to monitor platelet count, haemoglobin concentration, and packed cell volume.

Studies on Normal Volunteers (N = 7)

Platelet survival in vivo. Volunteers for this study were drawn from the laboratory staff and participated with fully informed consent and prior approval from the

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University and Hospital Ethics and Research Committee. The pH of approximately 100 ml of platelet-rich plasma was adjusted to 6.5 ± 0.2 by the addition of further ACD-A and incubated with 400 μCi of sodium radiochromate, according to the recommendations of the International Committee for Standardization in Hematology [14]; the efficiency of labelling under these circumstances is approximately 10%. Following injection of the radiolabelled platelets, 5-ml samples of whole blood were obtained from the opposite arm at intervals of 10 min, 1 hr, 3 hr, and daily thereafter for 8 days. The level of radioactivity in these samples was counted in a gamma spectrometer (Packard, Model 3820, IL) to a standard deviation of 1%. Platelet recovery was defined as the percentage of the injected platelet-bound radioactivity remaining in the circulation 10 min after injection and was derived from the formula:

\[
\text{Recovery} = \frac{\text{cpm/ml} \times \text{blood volume}}{\text{total radioactivity injected}}
\]

Blood volume values were derived from standard tables [15]. Platelet survival curves from the observed radioactivity data were used to calculate platelet mean life span using a linear function [16].

Platelet aggregometry. A control blood sample was obtained from donors at the start of the procedure and anticoagulated with ACD-A using the same ratio of blood to anticoagulant as that required for the apheresis procedure. This sample was immediately centrifuged to obtain platelet-rich and platelet-poor plasma. Platelet counts were determined by phase contrast microscopy [17] or the Coulter Counter (model ZF) or Thrombocounter-C (Coulter Electronics Incorporated, Hialeah, FL) [11]. The postapheresis sample was taken directly from the collection bag at the conclusion of the donation. No additional anticoagulant was added to the sample and the virtual absence of contaminating red cells made additional centrifugation unnecessary. These harvested platelets were counted and the level adjusted to correspond to the control sample by the addition of appropriate volumes of autologous platelet-poor plasma. The level of calcium was adjusted to 2.2 mmol/liter, where necessary, with calcium chloride.

Platelet aggregation was carried out by the method of Born and Cross [18], using the Payton Twin-Channel Aggregometer (Payton Associates Incorporated, Roswell Park, NY). The final concentration of the aggregating agents was ADP 10 μM, 100 μM, and 1 mM (Sigma Chemical Company, St Louis, MO), adrenaline 100 μM (Wyeth Laboratories, Philadelphia, PA), and collagen 7.5 mg/ml (Type V, Sigma Chemical Company, St Louis, MO). The effect of these agents was recorded as the maximum increase in light transmission after 8 min on a scale where platelet-rich plasma corresponds to 0% transmission and that for platelet-poor plasma to 100%. The individual results obtained from control and apheresis platelets, with each agent, were expressed as a mean, and the significance of the difference between the means assessed by the Students t test [19].

Platelet factor III availability. This was assayed using standard methods [20,21].

Ultrastructure. Samples of control and harvested platelets were concentrated by centrifugation at 200 g, fixed in phosphate-buffered gluteraldehyde, and postfixed in osmium tetroxide. The samples were block stained in 2% uranyl acetate in 10% acetone and then dehydrated with increasing concentrations of acetone and embedded in spars resin. Sections were prepared on a Reichert Ultratome (UM3) and stained with uranyl acetate and 0.2% lead citrate. The sections were viewed with a Siemens Electron Microscope (Model 101A).

RESULTS

Platelet Donations for Therapeutic Purposes

The mean procedure time was 87 ± 13 min and in no instance was this discontinued because of mechanical failure. In 35 patients, 11% mild hypocalcaemic symptoms developed, which were rapidly reversed after the infusion of 10 ml of 10% (w/v) calcium gluconate (Glaxo, England). A single patient developed transient vasovagal attack during the venipuncture.

The volume of blood processed was 4190 ± 533 ml (mean ± SD). The product volume in the collection bag was 238 ± 27 ml (mean ± SD). Packed red cell volume was 0, and contaminating leucocytes were 0.2 ± 0.30 × 10⁹/liter (mean ± SD).

The absolute platelet yield was 5.1 ± 1.45 × 10¹¹ (mean ± SD). The extraction efficiency, defined as the yield of platelets divided by the number presented to the machine, multiplied by the volume processed, was 62.11% and is therefore superior to the 51% reported for platelets harvested using the single channel module [7]. The platelet yield was significantly correlated with the volume processed (r = 0.89; p < 0.001) and the platelet precursor (r = 0.51; p < 0.01). There was no statistically significant difference in platelet yields between males and females (p > 0.05).

Donor platelet count fell from 265 ± 68.5 to 189 ± 46.3 (mean ± SD) (p < 0.01) and lymphocyte count from 2.1 ± 0.73 to 1.8 ± 0.67 × 10⁹/liter (mean ± SD) (p > 0.05). There were no changes demonstrable in red cell or absolute granulocyte count.

Infusion of platelets into previously unsensitized recipients with hypomegakaryocyte thrombocytopenia achieved a 1-hr increment of 19 ± 7.3 × 10⁹/liter/m²
(mean ± SD) and a 24-hr level of $15 \pm 6.3 \times 10^9$/liter/m² (mean ± SD).

Studies on Normal Volunteers

Platelet survival. The platelet recovery at 10 min was 59.4 ± 3.4% (mean ± SD) (normal 63.5 ± 4.2). The linear estimate of platelet mean life span was 218 ± 12 hr (mean ± SD) (normal 213 ± 15 hr) [16].

Platelet factor III availability. In the individual studies, maximum time differences between any of the tubes was 4 sec; no abnormal results were obtained (Table I).

Platelet aggregometry. Initially, suboptimal responses were obtained from platelets in the collection bag. In all instances this was immediately corrected by resuspending the platelets in control platelet-poor plasma. Further examination showed the abnormality to be related to a low calcium concentration of 1.83 mmol/liter and when this was corrected to 2.2 mmol/liter, response to standard aggregating agents was normal.

Ultrastructural studies. There was no difference demonstrable between platelets recovered from the apheresis collection bag at the end of the procedure and those obtained by venipuncture before its commencement.

DISCUSSION

Thrombocytopenic bleeding due to diminished platelet production can be controlled by the infusion of adequate numbers of functionally normal allogeneic platelets with the proviso that prior sensitization has not taken place [5,22,23]. The platelets may be obtained from single units of whole blood or collected on cell separators [23]. The latter machines are efficient. The yield from a single donor is comparable to that from six units of whole blood; it can be obtained in a reasonable period of time, and there is minimal risk or discomfort to the donor. A number of different separators are currently in use, including the Hemonetics [24,25], the IBM 2997 using the single channel module [7], and the Fenwal CS3000 [26].

A recent modification to the original single channel used with the IBM 2997 separator is known as the dual-channel module and has been specifically engineered to enhance platelet collection [9]. In this system flow is directed to result first in separation of red cells and thereafter platelets are recovered from the platelet-rich plasma by differential centrifugation. This unique design provides an efficient system for platelet harvesting so that a single procedure can be carried out in approximately 90 min and will result in an absolute mean platelet count of $5.1 \times 10^11$. Contaminating red cells and white cells are, for practical purposes, absent. The overall extraction efficiency has been raised to 62% from the 51% previously reported for the single channel used on the same separator [7]. A further point of distinction between the two channels is that the higher yields obtained from men using the single channel is not evident when the dual-channel module is employed. However, both platelet and lymphocyte depletion are of approximately the same magnitude as occurs with the single channel instrument [8], whereas red cell contamination was 2% with the single channel and absent using the dual channel.

The function of the harvested platelets as assessed by in vivo survival and in vitro platelet factor III availability and aggregometry did not differ significantly from normal control value. It was, however, of note that the platelets in the bag showed defective aggregation patterns that were related to suboptimal calcium concentrations, and this is consistent with minor degrees of hypocalcaemia occurring in 11% of our patients and also with the well recognized effects of citrate anticoagulation on the donor [27]. It is noteworthy that correction of the low calcium levels in the bag, either by titration into the normal range with chloride or resuspension of the platelet pellet in pretreatment autologous donor plasma, resulted in immediate return of function to normal. Furthermore, ultrastructural studies showed morphology to be indistinguishable from normal controls.

The postinfusion platelet increments in these patients who had not been previously sensitized, who were not bleeding, and who did not have clinically palpable spleens are less than previously reported figures of corrected posttransfusion increments of $75 \times 10^9$/liter/m² at 1 hr and $45 \times 10^9$/liter/m² at 24 hr [28,29] but are consistent with more recently described figures at 1 hr of $18.3 \pm 2.6 \times 10^9$/liter/m² and corrected 20-hr posttransfusion increment of $13 \pm 1.4 \times 10^9$/liter/m² [8]. This response was obtained in all but six of the patients receiving therapeutic donations. It should be pointed out that this study did not specifically address the question of whether there was any clinical benefit from these increments obtained by allogeneic platelet transfusion.

Plateletpheresis can be carried out with minimal discomfort to the donor and symptoms are restricted to transient and easily reversible hypocalcaemia. It is concluded that the dual-channel module provides a safe, simple, and efficient system for high yield collection of functionally normal platelets with negligible cellular contamination.

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ACKNOWLEDGMENTS

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REFERENCES


Exchange Transfusion in Sickle Cell Disease using a Continuous-Flow Blood Cell Separator

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An exchange transfusion was performed preoperatively on a patient with sickle cell disease using a continuous-flow blood cell separator. An exchange of 2,825 ml red blood cells achieved a hemoglobin A level of 90.8 per cent. The continuous-flow blood cell separator appears to offer a safe and effective method of exchange transfusion in sickling disorders.

Exchange transfusion is frequently of benefit to patients with sickling hemoglobins, but performed in the conventional way may be time consuming and relatively inefficient. The continuous-flow blood cell separator offers an alternative means of exchange transfusion, and the efficiency of this method was assessed in a patient with sickle cell disease who required exchange transfusion prior to surgery.

Case History

The patient, a 19-year-old female, was diagnosed as having sickle cell disease at the age of 15 months. The events leading to the diagnosis and her early medical history have been reported previously. Her subsequent growth and development was normal, and she remained in good health until she developed episodes of upper abdominal pain. The clinical diagnosis of cholelithiasis was confirmed by oral cholecystogram, and at operation numerous mixed gallstones and a small splenic remnant were found. The operation and postoperative course was uncomplicated, and she was discharged on the seventh postoperative day.

Method

Exchange transfusion was performed with an IBM Blood Cell Separator (Model 2990), four days before surgery. The system was primed with 280 ml of normal saline containing 5,000 units of heparin. Further heparin was infused during the exchange at a rate of 250 units per hour. The exchange procedure comprised two phases. Initially 300 ml of the patient's red blood cells were exchanged with normal saline, this being, in effect, a venesection. This served the purpose of reducing the amount of blood in the circulation which would dilute the incoming normal blood. Thereafter 2,825 ml of the patient's red blood cells were exchanged with an equivalent volume of normal red blood cells with a hematocrit of 80 per cent. The exchange procedure lasted 3.5 hours, being performed at a flow rate of 10 to 15 ml/minute, with the centrifuge bowl at a speed of 1,000 rpm. After completing the exchange, the patient received a transfusion of 750 ml of red blood cells.

Venous blood was obtained prior to commencing the exchange, and thereafter at regular intervals for the measurement of the hemoglobin concentration (Coulter Counter Model S) and hemoglobin components. Red blood cell lysates were prepared and hemoglobin electrophoresis on cellulose acetate performed according to Lehman and Huntsman. The electrophoretic fractions were eluted with distilled water and quantitated spectrophotometrically. Since HbF is not separated from HbS with this form of electrophoresis, HbF was quantitated by an alkali denaturation method.

Results

The change in hemoglobin concentration observed throughout the procedure is presented in Figure 1. The hemoglobin concentration fell from 8.0 g/dl to 6.3 g/dl after the 300 ml venesection, but rose to 11.8 g/dl by the end of the exchange. This rise in concentration during an exchange of
equal volume was accounted for by the higher hematocrit of the blood entering the system (80 per cent) than that being removed. Before the exchange the values for the hemoglobin components were: HbA 2.3 per cent; HbS + F 97.7 per cent; HbF (alkali denaturation) 11.4 per cent. After the 2825 ml exchange, HbS + F had fallen to 7.4 per cent; HbF to 2.4 per cent, and the HbA had risen to 90.8 per cent. After the 750 ml transfusion HbS + F fell to 4 per cent; HbF fell to 1.7 per cent, and HbA rose to 94.7 per cent. These changes are illustrated in Figure 2. At follow-up, 30 days later, the HbS + F was 89.9 per cent, HbF 4.0 per cent and HbA 8.1 per cent.

Discussion

The exchange transfusion described above was performed without any side effects or discomfort to the patient, and provided her with greater than 90 per cent of normal hemo-
moglobin. Because her veins were of poor caliber it was not possible to exchange at the usual speed of 40 ml/minute. Had this been possible, the time taken for the procedure could have been reduced by half. The level to which the hemoglobin rose was greater than anticipated and, in retrospect, the transfusion following the exchange was probably not necessary.

The indications for exchange transfusion in sickling disorders have been discussed elsewhere, and it is not suggested that these should be altered in the light of the present report. However, the increasing availability of the continuous-flow blood cell separator and the present demonstration that it can be used to perform a rapid, safe and effective exchange, commend this method to further trial under these differing clinical situations.

References

The Function and Structure of Granulocytes Collected Using the IBM 2997 Separator

Lucille Wood, Jeane P. Hester, and Peter Jacobs

The University of Cape Town Leukaemia Centre and The Department of Haematology, University of Cape Town and Groote Schuur Hospital, Observatory, Cape Town, South Africa (L.W., P.J.), and The M.D. Anderson Hospital and Tumor Institute, Houston, Texas (J.P.H.)

The effect of oral methylprednisolone and the sedimenting agent, hydroxyethyl starch, on granulocyte recovery, morphology, and function was studied in a volunteer donor programme. Using the IBM 2997, 10 litres of whole blood were processed, with an average procedure time of 2.4 hours and a collection volume of 300 ml. Donors not receiving methylprednisolone (n = 80) had a mean total granulocyte count of \(3.5 \times 10^9/\text{litre}\) (range 1.6-5.3 \(\times 10^9/\text{litre}\)) and mean granulocyte yields were \(1 \times 10^8\) (range 0.2-3.0 \(\times 10^8\)). Those receiving 48 mg oral methylprednisolone 6-8 hours before the procedure (n = 320) had a mean granulocyte count of \(6.3 \times 10^9/\text{litre}\) (range 3.2-11.4 \(\times 10^9/\text{litre}\)) and significantly superior mean granulocyte yields of \(2.0 \times 10^9\) (0.3-6.5 \(\times 10^9\)) (P < 0.05). For both groups the mean packed cell volume of 0.08 litre/litre (range 0.02-0.17) and platelet contamination 1.9 \(\times 10^{11}\) (range 0.3-5.0 \(\times 10^{11}\)). In all these procedures, hydroxyethyl starch was added to the blood entering the centrifuge channel. In none of the procedures were any untoward symptoms experienced by the donors. Light microscopy and ultrastructural studies showed no difference between control granulocytes and those collected following the addition of hydroxyethyl starch or after oral methylprednisolone. Similarly, granulocyte function measured with a random migration, chemotaxis, phagocytosis, and intracellular killing was not significantly different between control cells and those exposed to the sedimenting agent or the adrenocorticosteroids (P > 0.10). It is concluded that donor presedation with methylprednisolone significantly enhances granulocyte yields in the presence of hydroxyethyl starch and neither agent has any demonstrable effect on granulocyte morphology or function.

Key words: adrenocorticosteroids, hydroxyethyl starch, granulocyte structure and function

INTRODUCTION

Optimum granulocyte transfusions depend upon obtaining the largest number of functionally intact cells, and modern apheresis techniques are ideally suited to this purpose. Of the methods available, continuous-flow filtration has fallen into disrepute because of donor reactions [1-3] and evidence that the harvested cells may be morphologically [4] and functionally [5,6] abnormal. By contrast, centrifugal techniques are widely used, with optimal granulocyte yields being influenced by the type of instrument used, the duration of the procedure, and the initial white cell count of the donor. In an attempt to expand the circulating granulocyte compartment, donors have been treated with drugs which include eticholalone [7,8], dexamethasone [9,10], hydrocortisone, and prednisolone [11]. Efficiency is further enhanced by the use of sedimenting agents such as dextran or hydroxyethyl starch [12] to improve cell separation during centrifugation. Results reported with adrenocorticosteroids and hydroxyethyl starch are essentially similar to our earlier experience using the IBM 2990 cell separator [Wood L, Jacobs P: unpublished observations].

More recently there have been considerable technical improvements in the IBM series of separators, resulting in the production of a second generation instrument, designated the IBM 2997. In the new machine, the reusable polycarbonate bowl has been replaced by a single disposable plastic unit with separation taking place in a narrow channel. This technique was studied to define the effects of methylprednisolone, an adrenocorticosteroid which has not been extensively used in granulocyte collection, on donor counts and cell yields and to assess the effect of both this and hydroxyethyl starch on morphology and function of the harvested cells.

MATERIALS AND METHODS

Granulocyte Donors

Two groups of individuals were studied. The first were siblings of patients on a leukaemia or bone marrow transplantation programme where intensive granulocyte collection was carried out on 5 consecutive days. The second were random volunteers who donated with a max-

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Address reprint requests to Dr. Peter Jacobs, Department of Haematology, Research Centre, University of Cape Town Medical School, Cape Town, South Africa.
imum frequency of once every 4 months in view of the rare but recognized complications of hydroxystarch [13]. All donors fulfilled the standard requirements of the American Association of Blood Banks [14], being ABO-compatible and negative for hepatitis-B surface antigen and serologic evidence of syphilis. Participants were fully informed about the procedure and signed informed consent. In a control group (n = 80), white cells were collected without prior adrenocorticosteroid administration. In the test group (n = 320), donors took 48 mg oral methylprednisolone 6–8 hours before the procedure. This schedule was selected on the basis of donor convenience, effect on granulocyte mobilization [15], and confirmed by our own observation that this timing and dosage will consistently roughly double peripheral blood granulocyte counts.

Granulocyte Collection

All procedures were performed on the IBM 2997 continuous-flow cell separator. The collection line and channel were primed with 500 ml physiologic saline containing acid citrate dextrose (ACD-A) in the ratio of 13:1. Venous access was with a 16-gauge intravenous plastic cannula (Ethclor, South Africa) and flow rates varied between 40 and 80 ml/minute according to the donor’s lean body mass and surface area. The collection rate was between 1.8 and 3.3 ml/min and centrifuge speeds were 520–820 rpm. During the procedure blood was anticoagulated by infusing a solution containing 500 ml of 6% hydroxystarch (Fresenius, West Germany) and 62 ml of a concentrated citrate solution (18 g trisodium citrate and 1.8 g citric acid in 50 ml water) into the access line in a ratio of 1:18 with venous blood. Under these circumstances, uniform anticoagulation was obtained and 10 litres of blood processed without clinical or biochemical hypocalcaemia developing.

Haematological and Biochemical Measurements

Before and after each procedure, venous blood was collected for the measurement of haemoglobin, red cell count and indices, white cell count, and platelet count using the Coulter counter, model S-Plus [16], and differential count on a Romanowsky-stained film [17]. Biochemical profile, including urea and electrolytes, was similarly recorded [18]. Haemostasis [19] and changes in complement levels (C3 and CH50) were monitored, using standard techniques [20].

Special Haematology

Throughout the study additional samples were collected from 15 donors for morphologic and functional studies on the granulocytes. In these procedures a sample of blood was collected prior to oral methylprednisolone, a second 8 hours later immediately before commencement of the procedure, a third from the donor at the end of the collection after addition of hydroxystarch, and a final sample from the bag of harvested granulocytes.

Films were prepared using a blood spinning centrifuge [21] and stained with May-Grunewald Giemsa. Ultrastructural studies were carried out on gluteraldehyde-fixed material [22]. Granulocyte function was assessed by random migration and chemotaxis using a modified raft technique with 0.5% sodium caseinate as the chemoattractant [23–26]. Phagocytosis and intracellular killing were determined by liquid scintillation counting using a combined method which employed Candida albicans labelled with uridine ([5,6-3H] uridine: specific activity 40–60 Ci/m mol: Amersham, Buckinghamshire, England) as the target [27,28]. In this method, phagocytosis is determined by the loss of labelled uridine after cell harvesting and intracellular killing on the basis of isotope exclusion.

RESULTS

Donor Safety and Side Effects

None of the donors experienced side effects from the administration of adrenocorticosteroids or the hydroxystarch. Using this particular instrument and a strictly isovolaemic technique, three patients had transient discomfort and dizziness during venepuncture but in none of them did this require discontinuation of the cell collection. The mean total serum concentration at the commencement of the procedure was 2.6 mmol/litre and at its completion was 3.6 mmol/litre (range 2.1–2.6 mmol/litre) with a percentage change which was not statistically significant (P > 0.10). No changes in haematology or biochemical profile have been found. In no instance has regular follow-up revealed late effect from hydroxystarch accumulation.

Control Group

In the 80 donors who received no oral methylprednisolone premedication the starting total white cell count was 4.5 × 10⁹/litre (range 2.5–5.0), granulocyte count before the procedure was 3.5 × 10⁹/litre (range 1.6–5.3), and after cell collection was 2.5 × 10⁹/litre (range 1.3–4.5). Mean granulocyte yields were 1.0 × 10¹⁰ (range 0.2–3.0).

Adrenocorticosteroid Pretreated Donors

In the 320 donors who received 48 mg of oral prednisolone 6–8 hours before commencing cell collection, the starting total white cell count was 7.0 × 10⁹/litre (range 2.9–16.3), granulocyte count before the procedure was 6.3 × 10⁹/litre (range 3.2–11.4), and after cell collection was 4.6 × 10⁹/litre (range 1.6–9.4). Mean granulocyte yields were 2.0 × 10¹⁰ (range 0.3–6.5).
Red Cell and Platelet Contamination

For both groups the mean packed cell volume was 0.08 litre/litre (range 0.015–0.17) and platelet contamination $1.9 \times 10^{11}$ (range 0.3–5.0).

Morphology

In neither the control ($n = 8$) or the methylprednisolone-pretreated ($n = 15$) donors were any of the cells abnormal when examined by light or transmission electron microscopy. Specifically, nuclear chromatin, presence of vacuoles, appearance of cytoplasmic projections, or abnormal granule distribution were not affected by the cell collection procedure.

Granulocyte Function Studies

The serial investigation of random migration, chemotaxis, phagocytosis, and intracellular killing showed no effect as a result of either methylprednisolone administration or the subsequent exposure to hydroxyethyl starch during the collection technique in randomly studied patients ($n = 15$) (Table I).

Recipient Reactions

Infusion of allogeneic granulocytes to neutropenic patients was associated with a pyrexial reaction of varying severity in 26%. Premedication with 4 mg Chlorotrimeton (Essex) given orally half an hour before the cells reduced the incidence of this reaction to under 10%. It is, however, not clear whether this difference is significant since there is no sound reason why an antihistamine should prevent or modify fever. In those individuals where further granulocyte infusions were necessary and reactions persisted they could be blocked by the intravenous infusion of 100 mg Cortisol or 120 mg Paracetamol half an hour before commencing the granulocyte transfusion.

DISCUSSION

Although debate continues on the role that granulocyte transfusion should occupy in current clinical practice [29–31], and such issues as optimal diagnostic technique [32,33] or noninfectious pyrexia [34] are unresolved, there is little debate that, once committed to this form of treatment, the largest number of functionally intact granulocytes should be infused. These can be collected by modern techniques but filtration is now less favoured because of donor reactions [1–3] and evidence of abnormal cell morphology and function [4–6]. By contrast, centrifugation is being increasingly used and attempts to enhance recovery have led to modifications of the instrument, variations in the duration of the procedure, and use of manipulations to raise white cell count in the donor coupled with sedimenting agents to enhance or improve the efficiency of cell separation. We have examined two of these variables, using a standardized collection procedure and the single channel module for the IBM 2997 continuous-flow blood fraction separator.

First, pretreatment of donors with adrenocorticosteroids significantly raised starting total white cell and granulocyte counts and consequently doubled the number of cells harvested. Of the agents known to produce neutro-

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<th>TABLE I. Granulocyte Function Studies*</th>
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*In vitro comparison of cells from donor to those exposed to steroids alone or both steroids and hydroxyethyl starch shows no significant difference.
philic leucocytosis in man [7] the effects on mobilization and function of neutrophils have been characterized for hydrocortisone, prenisolone, and dexamethasone [11]. The choice of methylprednisolone in this study was based on the intermediate duration of biological activity over the shorter-acting hydrocortisone and the longer-acting dexamethasone together with the fact that it is essentially devoid of mineralocorticoid activity as opposed to prednisolone [36]. It is, however, unlikely that the magnitude of change produced by this agent is significantly different to that which could be achieved with equivalent doses of other analogs. Since the latter point was not specifically examined, we are unable to recommend our regimen over that used by other investigators: we have, however, found it a convenient and effective means for raising granulocyte counts in the peripheral circulation. Since this is a volunteer programme it is important to record that no complications such as changes in electrolyte status or blood sugar level were documented either following single or multiple exposures.

Second, the benefits of hydroxyethyl starch [36] have been confirmed. Furthermore, while adverse effects, including pruritus, have been reported [13], the accumulation of this agent in the reticuloendothelial cells from which it is then lost by subsequent slow metabolic degradation appears to be without deleterious side effects to the donor. Nevertheless, it is prudent to limit the number of procedures that volunteers undergo on the basis of currently available information on metabolism and clearance rates [36]. Thus, while no recommendations appear as yet to have been formulated, a donor panel sufficiently large to require a maximum of one procedure every 4 months is our established practice.

In a series of pilot studies, neither adrenocorticosteroid premedication nor hydroxyethyl starch infusion, when used separately, affected the morphology or function of the collected granulocytes but suboptimal cell yields were obtained. The present study extends these observations since it is known that the adrenocorticosteroids have wide-ranging effects on granulocytes [37-39]. In studies carried out on randomly selected donors, in which aliquots of cells were compared at different stages throughout the procedure, the combined effect of this pharmacological agent and hydroxyethyl starch were shown to be without recognisable effect on morphology or ultrastructure: a finding in keeping with other studies [4]. Similarly, no functional abnormality was demonstrated in the harvested granulocytes that had been exposed to methylprednisolone and the sedimenting agent, also in keeping with previous observations [40-43].

It is of interest that, although not part of the present study, we have previously shown in a small number of patients (n = 5) that bactericidal capacity of granulocytes is not affected by exposure to steroids using a coagulase-positive Staphylococcus aureus cell suspension [42]. Our current findings are in contrast with an earlier report [44] but are difficult to compare to that study because different assay techniques were employed.

CONCLUSION

It is concluded that donor premedication with methylprednisolone substantially enhances the circulating white cell count at the time of the collection procedure and, in the presence of hydroxyethyl starch, doubles the granulocyte yield. Neither adrenocorticosteroid nor the sedimenting agent, singly or in combination, had any demonstrable effect upon cellular morphology, ultrastructure, or function. It is therefore possible to endorse the recommendation that these two pharmacologic agents be combined in routine procedures to harvest donor granulocytes for transfusion. Although of infrequent occurrence, adverse effects attributed to the sedimenting agent may be disturbing to the individual donor and, therefore, an urgent need exists to examine alternatives to hydroxyethyl starch. It is also clearly necessary to formulate guidelines for donor safety, including a clear definition of currently acceptable ethical practice.

ACKNOWLEDGMENTS

This study was supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the Medical Research Council, the National Cancer Association, and the Kaliski and Michael Chanani bequests. We thank Jackie Davies and Dorothy Banner for typing, Jeanne Walker for illustrations, Sheila Katcher for bibliographic assistance, and the Medical Superintendent, Groote Schuur Hospital, for permission to publish. The granulocyte function studies were carried out by Wendy Paulsen and Ri Schneider and the electron microscopy was done by John Horne.

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Collection and Cryopreservation of Human Stem and Progenitor Cells for Bone Marrow Transplantation

Peter Jacobs, Lucille Wood, and Sue Horak

The University of Cape Town Leukaemia Centre and The Department of Haematology, Groote Schuur Hospital, Observatory, Cape, South Africa

Bone marrow collection was undertaken from human organ donors (Group 1; n = 7) to develop a closed-system single-step technique for stem and progenitor cell enrichment, using the Cobe 2997 continuous-flow blood-cell separator. The effects of programmed freezing, storage in liquid nitrogen, and thawing were then defined using these grafts. Once standardized, this method was extended to autografting following cryopreservation of a comparable fraction (Group 2; n = 8) and then to allogeneic transplantation after ex vivo exposure to the lytic monoclonal antibody, Campath-1 IgM and human complement, but without cryopreservation (Group 3; n = 9). The median number of mononuclear cells harvested was 5.0 × 10^6/mL (n = 24), and this was not significantly different in the three groups. The ex vivo graft, composing marrow rich antiagglutinated whole blood, was recirculated in the separator at a flow rate of 60 mL/minute, with a centrifugation speed of 1,100 r.p.m., and the mononuclear cell fractions were collected at the rate of 1.5 mL/minute. The average procedure time from formation of the interface in the single disposable channel to achievement of the final volume was 90 minutes. The mean recovery of the mononuclear cells was 101.4% (SD 38.0) and the GM-CFUc was 91% (SD 43.86). These figures were not significantly influenced by subsequent cryopreservation (Group 1; n = 7 and Group 2; n = 8) or following exposure to the monoclonal antibody, Campath-1 IgM (Group 3; n = 9). The Cobe Model 2997 continuous-flow blood fraction separator is ideally suited for the ex vivo enrichment of human bone marrow stem and progenitor cells in a volume that makes handling practical by a technique that is rapid and readily applicable to both autografting and allogeneic transplantation programmes.

Key words: stem cell harvesting, continuous flow separator, Cobe 2997, CAMPATH-1

INTRODUCTION

Bone marrow transplantation is of increasing importance in clinical practice. The well-established technique for allogeneic grafting is constantly being refined by ex vivo procedures that include exposure to monoclonal antibodies [1] or cytotoxic agents [2], aimed at reducing the incidence and severity of graft-versus-host disease. However, because the availability of suitable donors is limited, autologous transplantation is rapidly being developed, and here interest centres on the use of physical and immunologic manipulations to selectively remove contaminating malignant cells and thereby reduce disease relapse [3]. In both of these procedures, there are three practical considerations. Firstly, it is necessary to achieve maximum recovery of stem and progenitor cells to ensure that adequate numbers remain after any ex vivo graft manipulation to reliably reconstitute haematopoietic and immunologic function. Secondly, the enriched population should be in a reasonably small volume of autologous plasma to limit the space required for graft storage following cryopreservation and prior to subsequent reinfusion. Thirdly, where exposure to monoclonal antibodies is employed, it is critical to standardize the ratio of these reagents to target cells, and this is achieved most easily where the relevant population is available in the concentrated preparation. Several methods have been described to selectively harvest the mononuclear cell population, including manual techniques of double centrifugation [4], density sedimentation [5], Amino-1 [6], and Hemogenics [7,8] separators, and the IBM 2991 red cell processor [9]. Recently, data has been presented for the Cobe 2997 [10–13], and we report additional and confirmatory experience with this instrument, including studies to characterise the behaviour of the enriched population following cryopreservation and ex vivo manipulation with monoclonal antibodies.

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MATERIALS AND METHODS

In a feasibility study (Group 1; n = 7), bone marrow was collected as the first procedure from organ donors at a time when full cardiovascular and endocrine support was maintained [14]. This approach is easier than the previously reported method [15] and approximates the established clinical techniques. All procedures were carried out with informed consent on programmes approved by the Ethics and Research Committee at the University of Cape Town and Groote Schuur Hospital. Once the preliminary studies had established the detailed methodology, it was extended to currently active autograft (Group 2; n = 8) and allograft programmes (Group 3; n = 9).

A standard approach was used in which marrow-rich blood was aspirated from the sternum and both the anterior and posterior iliac crests under sterile conditions in an operating theatre [16]. Collection took place into plastic syringes containing McCoy’s tissue culture medium (TCM) with a final concentration of 30 i.u./mL preservative-free heparin (Polarin, Evans Medical Limited, Langhurst, Horsham, England). The ratio of TCM to marrow was 1:10. After measurement of total volume and removal of a representative aliquot to determine cellularity, absolute mononuclear count, and GM:CFUs, the bag of marrow-rich blood was suspended and recirculated through a Cobe 2997 blood fraction separator, with a flow rate of 60 mL/minute and a centrifuge speed of 1,100 r.p.m. The single disposable band was primed with physiologic saline, and ACD-A was added to the marrow in the ratio of 1:9 until a total volume of 400 mL had accumulated in the system. From the time that the interface became established, a mean procedure time was 90 minutes and the mononuclear cell fraction was collected at a rate of 1.5 mL/minute into a final volume between 120 and 150 mL of autologous plasma and heparin-containing TCM. In this technique, volumes as low as 500 mL could conveniently be processed.

Following completion of the procedure, triplicate counts on both the collection and discard bags were carried out to determine the mononuclear cell fraction in each. Specifically, there should be quantitative recovery of this fraction and less than 10% of the original number of monocytes and lymphocytes should be present in the discard bag. The contents of the latter was reinfused into the donor, if necessary with diuretic cover, to avoid fluid overload.

In the organ donor and autograft programme, cryopreservation was undertaken by adding dimethylsulphoxide (DMSO). The freezing medium consisted of 20% of autologous plasma and 20% DMSO in tissue culture medium, so that the final concentration of the cryoprotectant was 10%. The harvested cell fraction was transferred to freezing bags (Delmed: Type 3200-2), and the temperature was reduced in a freezing chamber coupled to a programme controller (Union Carbide Corporation, Linde Division, New York) in which the rates and settings for the machine were predetermined to give a freezing curve of −1°C/minute down to −40°C and −2°C/minute down to −80°C. The temperature characteristics of each procedure were monitored, using a chart recorder (Esterline Angus Instrument Corporation, Indiana). The bag containing the marrow was then stored in the fluid phase in a liquid nitrogen refrigerator (Union Carbide Corporation, Linde Division, New York). When required for infusion, the unit was thawed at the patient’s bedside in a 37°C water bath and administered without a filter. Since small amounts of fibrin and cell debris were present, these were retained by allowing them to settle into a corner during this procedure simply by holding the bag at an angle of 45°. The final volumes of DMSO infused into the patients varied between 24 and 34 mL, and side effects were infrequent but included hot flushes, an unpleasant taste in the mouth, and a strong smell of the agent around the patient for approximately 12 hours.

In the current allograft programme, T-lymphocyte depletion was undertaken using the lytic monoclonal antibody Campath-1 IgM combined with human complement as part of a collaborative study [17] and marrow was reinfused without cryopreservation. The efficacy of the ex vivo manipulation was quantitated and expressed in absolute cell numbers and residual T-cells defined using sheep rosette formation.

In all these studies, GM:CFUs was determined, using a standard assay [18], on mononuclear cells that had been separated on a Ficoll-Hypaque density gradient (Lymphoprep, Nycomed AS, Oslo, Norway; specific gravity 1.077 g/mL) using aliquots derived from the original marrow-rich sample. The in vitro culture studies were repeated on the enriched population after separation, following T-lymphocyte depletion, or after the rapid thawing of the cryopreserved units.

RESULTS

Marrow harvesting from organ donors, patients for autografting, and siblings for allogeneic transplantation were equally effective (Table 1). Although mean volumes as well as total nucleated cells varied, their concentration in the three groups was comparable (P > 0.05). Similarly, the concentrations of the total mononuclear cells, determined by differential counting, did not differ significantly (P > 0.05). Cell viability on all these fractions was the same and approximated 100%.

Cell viability was determined by trypan blue exclusion. Equal volumes of 0.73% trypan blue were mixed
TABLE I. Cellular Composition of Marrow-Rich Blood Collected From the Donors

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>527.1</td>
<td>1028.7</td>
<td>1108.8</td>
</tr>
<tr>
<td>S.D.</td>
<td>298.3</td>
<td>156.7</td>
<td>158.9</td>
</tr>
<tr>
<td>Range</td>
<td>228.8–825.4</td>
<td>872.0–1185.4</td>
<td>949.9–1267.7</td>
</tr>
<tr>
<td>Nucleated cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (×10⁶)</td>
<td>1.0</td>
<td>2.06</td>
<td>3.02</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.8</td>
<td>0.55</td>
<td>0.91</td>
</tr>
<tr>
<td>Range</td>
<td>0.2–1.8</td>
<td>1.51–2.61</td>
<td>2.11–3.93</td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (×10³/mL)</td>
<td>1.80</td>
<td>1.95</td>
<td>2.7</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.57</td>
<td>0.64</td>
<td>0.63</td>
</tr>
<tr>
<td>Range</td>
<td>1.23–2.37</td>
<td>1.31–2.59</td>
<td>2.07–3.33</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (×10⁶)</td>
<td>1.93</td>
<td>4.0</td>
<td>7.73</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.53</td>
<td>1.33</td>
<td>2.22</td>
</tr>
<tr>
<td>Range</td>
<td>1.40–2.46</td>
<td>2.67–5.33</td>
<td>5.51–9.95</td>
</tr>
<tr>
<td>Concentrations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (×10⁴/mL)</td>
<td>2.85</td>
<td>3.98</td>
<td>6.90</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.99</td>
<td>1.54</td>
<td>1.65</td>
</tr>
<tr>
<td>Range</td>
<td>1.86–3.84</td>
<td>2.44–5.52</td>
<td>5.25–8.55</td>
</tr>
</tbody>
</table>

S.D. = standard deviation.

with 1.8% sodium chloride, and equal volumes of this mixture were added to the cell suspension; non-viable cells took up the dye-staining blue.

In vitro GM:CFUc for an aliquot collected from the graft prior to manipulation and expressed in colonies and clusters was 22.36; 38.87; and 78.113, respectively. When corrected for the mean total volume harvested, there was no difference between these three sources of committed progenitors; the median was 5.0 × 10⁵ (S.D. = 2.7; range 2.3–7.7).

Continuous-flow separation (Table II) gave comparable results for final collection volumes and mononuclear cell recovery. There was no difference between the three groups for mean pre-separator red cell, granulocyte, and platelet count, and similar reduction in each of these formed elements was evident after the procedures. The reduction in mean haemoglobin from the marrow-rich blood to the stem cell concentrate was 85%, with no significant change in the total number of white cells, but the composition changed with granulocytes falling from 80 to 20%, lymphocytes being enhanced from 18 to 60%, and platelet concentration increased approximately threefold following the separation procedure.

Post-separator GM:CFUc recovery was a mean of 82%, with the three groups being 60%, 93%, and 93%, respectively; there was no loss of viability on trypan blue testing.

Cryopreservation resulted in an average loss of viability on using trypsin blue exclusion of 13.6%. In contrast, the GM:CFUc recovery was at a mean of 105%, with a standard deviation of 40.2. The respective figures for cadavers (Group 1) were 104.7% (SD = 20.4; range 84.3–125.1), for autografts (Group 2) 72.3% (SD = 22.7; range 49.6–95.0), and allografts (Group 3) 123.1% (SD = 54.3; range 68.8–177.4); these results are not significant (P > 0.05).

Following ex vivo exposure to Campath-1 IgM, viability could not be assessed because of extensive cell death due to the effect of antibody and complement. In these studies, mean residual T-cells were 0.54% (SD = 0.36; the numbers infused were 6.13 × 10⁷/kg (SD = 4.84; range 1.29–10.97).

In Groups 2 and 3, the rate of post-transplantation haematopoietic reconstitution was comparable. Thus, with regular monitoring of the peripheral blood count, the median time to reach 1 × 10⁹/L granulocytes was 21 days, and to achieve an unmaintained platelet count > 50 × 10⁹/L, 30 days.

DISCUSSION

The enrichment of stem and haematopoietic progenitor cells in human grafts by ex vivo techniques is possible, using manual methods [4,5] or by processing the marrow-rich blood on one of the different cell separators [6–10] currently available. Corresponding data for the Cobe 2997 have been reported for a dog model [11], and, recently, the earlier studies on this instrument have been expanded from three French centres [12,13]. We report further data on using this instrument that confirms its eminent suitability for procuring haematopoietic stem and progenitor cells for human transplantation programmes. This technique continues to undergo improvement in two major directions.
TABLE II. Recovery Studies Following Continuous-Flow Separation

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (mL)</td>
<td>133</td>
<td>150</td>
<td>167</td>
</tr>
<tr>
<td>S.D.</td>
<td>13.76</td>
<td>19.29</td>
<td>27.8</td>
</tr>
<tr>
<td>Range</td>
<td>120–147</td>
<td>131–169</td>
<td>83.9–12.7</td>
</tr>
<tr>
<td><strong>Pre-separator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total nucleated cells $\times 10^9$/kg</td>
<td>1.37</td>
<td>3.23</td>
<td>4.77</td>
</tr>
<tr>
<td>S.D.</td>
<td>1.12</td>
<td>0.73</td>
<td>1.36</td>
</tr>
<tr>
<td>Range</td>
<td>0.25–2.49</td>
<td>2.5–3.96</td>
<td>3.41–6.13</td>
</tr>
<tr>
<td>Mononuclear cells $\times 10^9$/kg</td>
<td>0.25</td>
<td>0.58</td>
<td>1.15</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.05</td>
<td>0.12</td>
<td>0.32</td>
</tr>
<tr>
<td>Range</td>
<td>0.2–0.3</td>
<td>0.38–0.78</td>
<td>0.83–1.42</td>
</tr>
<tr>
<td><strong>Post-separator</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells $\times 10^9$/kg</td>
<td>0.3</td>
<td>0.6</td>
<td>1.1</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.25</td>
<td>0.2</td>
<td>0.36</td>
</tr>
<tr>
<td>Range</td>
<td>0.05–0.55</td>
<td>0.4–0.8</td>
<td>0.74–1.46</td>
</tr>
<tr>
<td>Mononuclear cells $\times 10^9$/kg post T-cell depletion</td>
<td>—</td>
<td>—</td>
<td>0.76</td>
</tr>
<tr>
<td>S.D.</td>
<td>—</td>
<td>—</td>
<td>0.4</td>
</tr>
<tr>
<td>Range</td>
<td>—</td>
<td>—</td>
<td>0.36–1.16</td>
</tr>
</tbody>
</table>

S.D. = standard deviation.

Firstly, since autologous transplantation is being increasingly used in human cancer therapy [19], it is necessary to be certain that the choice of cryopreservative agent, freezing techniques, and storage characteristics do not damage the graft. It has been suggested that freezing rates may be critical when monitored by recovery in bone marrow culture studies [20]. We used in vitro clonogenic assays as previously described for predicting engraftment following allogeneic transplantation [21] to monitor this procedure, and it is notable that there was quantitative recovery of these cells in the GM:CFUc assay in Groups 1 and 2. Furthermore, in Group 2, the time taken after autografting to achieve $1 \times 10^9$/L granulocytes or $> 50 \times 10^9$/L platelets is consistent with that previously reported using other instruments [14,22] without cryopreservation, thereby confirming that manipulations do not damage the subpopulation. Of interest is the recent observation that unfractionated marrow which was cryopreserved without the use of programmed freezing gave essentially comparable results [23], suggesting that these procedures could be used in centres lacking expensive instruments.

Secondly, the enriched population was found to be convenient for ex vivo treatment of the monoclonal antibody, Campath-1 IgM, as previously suggested for antibody mixtures or other forms of purging [10]. Using Campath-1 IgM, where cell lysis was effected with human complement, viability was difficult to determine with trypan blue because of the confounding influence of extensive cell death. However, efficient removal of T-cells, determined using both sheep rosette formation and appropriate monoclonal antibodies, was consistently in excess of 99%. After this procedure, overall loss of nuclear cells was small and between 0.59 and $7.45 \times 10^7$ were infused, which corresponds to between 1.29 and $10.97 \times 10^7$ cells/kg. When this figure was extrapolated to GM:CFUc recovery, the fraction should have had the same repopulating potential as the mononuclear cells that had not been exposed to the monoclonal antibody. The validity of this hypothesis, based on the in vitro culture assay, is found in the mean time taken to establish $1 \times 10^9$/L granulocytes or a platelet count $> 50 \times 10^9$/L, being no different from historical controls [24] or in the patients in Group 2 who were autografted without ex vivo manipulation.

It is concluded that the Cobe model 2997 continuous-flow blood fraction separator is well suited for ex vivo enrichment of human bone stem and progenitor cells by recirculation of the marrow-rich blood collected during the harvesting procedure. This technique makes it feasible to rapidly concentrate the desired population in small volumes of autologous plasma, which is then readily applicable to both autografting and allogeneic transplantation programmes.

ACKNOWLEDGMENTS

We thank Professor Bruno Reichart, Dr. Dmitri Novitzky, Professor David Cooper, Dr. Jack Jacobson, and Sister Maryna Meyer for permission to collect marrow from human organ donors, Keren Houssell for bibliographic assistance, and Jackie Davies for typing and help with preparation of the manuscript.
REFERENCES


Aluminium Loading During Therapeutic Plasma Exchange

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The University of Cape Town Leukaemia Centre and the Departments of Pharmacology (F.M., P.F.), Haematology (L.W., P.J.), and Medicine Renal Clinic (M.C.), Groote Schuur Hospital, Observatory, Cape, South Africa

Graphite furnace atomic absorption spectroscopy was used to demonstrate a fivefold increase in the median plasma aluminium levels of four patients undergoing therapeutic plasmapheresis. This plasma aluminium loading resulted from contamination of albumin incorporated in the replacement fluids and took place during its preparation, with albumin aluminium levels of 646, 669, and 715 μg/liter present in three representative samples. Aluminium overload may result in the development of encephalopathy and osteomalacia in patients with renal impairment, indicating the need to monitor aluminium levels in the albumin-containing replacement fluids to avoid this potential hazard.

Key words: aluminium overload, albumin contamination

INTRODUCTION

The clinically relevant sources of aluminium contamination include the water used in haemodialysis, oral antacids given as dietary phosphate binders, and solutions administered in total parenteral nutrition [1–3]. The adverse effects of pathological increases in this element include a progressive severe encephalopathy and osteomalacia [3]. It is known that replacement solutions used for plasma exchange may contain large quantities of aluminium [4], and albumin has been incriminated as the source of this contamination [5] as a result of accumulation of the element by protein during the filtration process employed for its purification [6]. To further define the potential for aluminium loading four patients were serially studied whilst undergoing therapeutic plasma exchange, with measurements carried out on the plasma before and after each procedure, assay of the exchange fluid, and separate analysis of the electrolyte used in preparation of the replacement solution.

MATERIALS AND METHODS

Plasma Exchange

In a standardised procedure 1.5 times the calculated plasma volume was exchanged in a strictly isovolaemic procedure by using a vein-to-vein circuit on the IBM 2997 Cell Separator. The replacement fluid used was made up of 800 ml balanced electrolyte solution (Pamlayte B, Sabax Limited, South Africa) to which was added 200 ml of a 20% human serum albumin obtained from a single source (Western Province Blood Transfusion Service, Cape Town, South Africa).

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a P-E 56 Recorder. Pyrolytically coated graphite tubes were used at all times.

The spectrometer was calibrated by setting the aluminium hollow cathode lamp wavelength to 303.9 nm, slit 0.7 nm, lamp 9 mA. Standard operating conditions for drying, ashing, atomising, and cleaning were: drying 120°C for 35 seconds, ashing 1,200°C for 25 seconds, atomising 2,700°C for four seconds, and cleaning 2,700°C for four seconds, respectively. A sample volume of 10 μl was used and assays performed in duplicate; calibration was done by the method of additions.

CASE REPORTS (Table I)

Prior to the study informed consent was obtained from the patients referred for therapeutic plasma exchange. Renal function was normal apart from the fourth patient, in whom biochemical measurements deteriorated following a gastric haemorrhage a few days prior to the exchange. None of the individuals had been on aluminium-containing medications apart from the latter patient who received these for his haematemesis. No clinical evidence of aluminium toxicity was noted in short term follow-up.

RESULTS

The results are detailed in Table II.

The median serum aluminium levels before and after the exchange procedure rose from 8 μg/liter to 42 μg/liter. The major source of this loading was from contaminated albumin used in preparation of the replacement fluid.

COMMENT

The adverse effects of excess aluminium are known [3]. Concern about the potential toxicity has revealed new ways in which this ubiquitous element may gain entry into the body [1,5]. Replacement solutions used in plasma exchange have been shown to contain significant amounts of aluminium [4] in which the major source of contamination is the albumin added to maintain oncotic pressures. This procedure may be associated with significant increases in the serum aluminium [5], and a fivefold increase was found in the present study, confirming these observations. This contamination was traced to the albumin used in preparing the replacement solution, with the albumin aluminium levels being in the previously described range [5]. During the preparation of the replacement solution in the pharmacy aluminium contamination was avoided, apart from use of the metal needle for mixing the solutions and from the glass bottles in which the albumin and electrolyte solutions were supplied.

Cautions should be exercised in the preparation of replacement fluids for therapeutic plasma exchange. Particularly when this procedure is to be used repetitively or when renal impairment is present. In these situations the body burden of this element may be increased to potentially toxic levels, suggested to be serum levels between 100 and 150 μg/liter [3]. In addition, patients may also be exposed to the hazard of aluminium loading from dietary phosphate binders and the use of other intravenous fluids [1,2]. In this study only a single source of albumin was examined and as a result of these findings, the local producer is to modify the filtration technique used in isolating and concentrating this protein to avoid the aluminium contamination. However, it remains theoretically possible that this may be a much more widespread problem [6] and it merits the attention of centres using plasma exchange.

This report is based on observations from single exchange procedures and only short-term observations are reported; long-term follow-up on our patients is not available. Nevertheless, there are many situations where plasma exchange is performed repetitively and such individuals may be exposed to the hazard of aluminium loading should the exchange fluids be prepared with contaminated albumin. Paradoxically, it has also been

<p>| TABLE II. Aluminium Levels in Plasma and Exchange Fluid |</p>
<table>
<thead>
<tr>
<th>Sample</th>
<th>Aluminium (μg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preexchange serum (n = 4)</td>
<td>8</td>
</tr>
<tr>
<td>Median</td>
<td>7</td>
</tr>
<tr>
<td>Range</td>
<td>110</td>
</tr>
<tr>
<td>Postexchange serum (n = 4)</td>
<td>42</td>
</tr>
<tr>
<td>Median</td>
<td>144</td>
</tr>
<tr>
<td>Range</td>
<td>48</td>
</tr>
<tr>
<td>Exchange fluid (n = 18)</td>
<td>72</td>
</tr>
<tr>
<td>Median</td>
<td>50</td>
</tr>
<tr>
<td>Range</td>
<td>132</td>
</tr>
<tr>
<td>Electrolyte solution (n = 3)</td>
<td>12</td>
</tr>
<tr>
<td>Albumin (n = 3)</td>
<td>646</td>
</tr>
<tr>
<td>Albumin</td>
<td>669</td>
</tr>
<tr>
<td>Citrate (n = 1)</td>
<td>715</td>
</tr>
</tbody>
</table>

<p>| TABLE I. Details of the Four Patients Studied |
|--------|------------------|</p>
<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>M</td>
<td>Unstable angina; coronary artery disease; type II familial hyperlipoproteinaemia</td>
</tr>
<tr>
<td>40</td>
<td>M</td>
<td>Immunologically mediated sensory-motor polyneuropathy</td>
</tr>
<tr>
<td>40</td>
<td>F</td>
<td>IgG myeloma; hyperviscosity syndrome</td>
</tr>
<tr>
<td>78</td>
<td>M</td>
<td>IgG myeloma; hyperviscosity syndrome</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gastric haemorrhage two weeks prior to exchange</td>
</tr>
</tbody>
</table>
shown that plasmapheresis can remove aluminium from the bodies of patients with severe renal failure and dialysis encephalopathy, although such reductions did not alter the course of the disease [4]. Further controlled studies will be necessary to resolve this latter issue.

It is concluded that aluminium contamination of replacement fluids used for plasma exchange is a potential source of toxicity, particularly where serial procedures are undertaken. Attention should be directed to this possibility and the albumin added to these solutions should be screened for aluminium content.

ACKNOWLEDGMENTS

This work was supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the National Cancer Association, and Medical Research Council. We acknowledge the assistance of Professor M. Orren and thank Jackie Davies for typing.

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Lack of Short-Term Effects on the Donor During Continuous-Flow Selective Mononuclear Cell Collection

Peter Jacobs and Lucille Wood

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In 50 individuals, intensive harvesting of relatively pure mononuclear cell fractions from the peripheral circulation was carried out in 198 procedures. Serial collections from bone marrow donors (group 1: \( n = 35 \)) or isolated procedures from volunteers (group 2: \( n = 15 \)) were without morbidity. A median yield of \( 4.0 \times 10^7 \) mononuclear cells were recovered in a final volume of 104 ml of cell-rich plasma, for which 4,300 ml of venous blood was processed in 107 minutes. In neither group were changes documented in donor white cell count or lymphocyte numbers. In group 1, a statistically significant but clinically unimportant transient fall occurred in the platelet count at the end of the 3-day intensive schedule. It is concluded that mononuclear fractions can efficiently be collected from normal donors without the development of relevant cell depletion.

Key words: continuous-flow centrifugation, biologic reagents, allogeneic marrow boosting

INTRODUCTION

Blood fraction separators were introduced for platelet [1] and granulocyte [2] collection. More recently, they have been increasingly used to facilitate plasma exchange for diseases thought to have an immunologic basis [3-5], while commercial applications include efficient procurement of blood fractions and components. Modern apheresis techniques can also be applied to the efficient collection of buffy layer or relatively pure mononuclear cell fractions from the peripheral blood. This technique is in clinical use to facilitate engraftment following bone marrow transplantation [6], while lymphocyte depletion procedures are employed to modulate the course of patients with severe acute aplastic anaemia [7,8]. Such relatively pure populations are also useful as biological reagents to standardise lymphocyte function tests and as a source of committed progenitor cells for in vitro marrow culture studies. The effect of the collection procedure on the donor is an important consideration and has been defined by studying in vivo the response of 50 individuals during mononuclear cell collection procedures.

MATERIALS AND METHODS

All participants were screened and accepted according to standard guidelines [9]; informed consent was obtained in each case. The bone marrow donors (\( n = 35 \)) underwent collection procedures for 5 consecutive days, commencing 24 hours after marrow harvesting. The volunteers (\( n = 15 \)) were drawn from a panel established to provide allogeneic platelets and granulocytes for a leukaemia and transplant programme; each specifically agreed to the extra donation for mononuclear cell collection.

The apheresis procedure was carried out on the IBM 2997 separator employing the single channel and with centrifuge speeds at 820 rpm; anticoagulation of venous whole blood was achieved with acid-citrate-dextrose at a ratio of 11:1. The medium value for blood flow was 40 ml/min and for cell-rich plasma collection was 1.5 ml/min. The volume of blood processed was 4,300 ml, resulting in a collection volume of 104 ml of cell-rich plasma over 107 minutes. To safeguard the donors, one of the investigators (L.W.) initiated the procedure and was present throughout. The patients received 10 ml of calcium gluconate half way through the procedure.

Haematologic measurements in the donors were carried out before, immediately after the procedure, and 24 hours later. These included full blood count [10], differential count [11], platelet count [12], biochemical profile [13].

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TABLE I. Haematologic Changes In Vivo—Bone Marrow Donors

<table>
<thead>
<tr>
<th></th>
<th>Preprocedure</th>
<th>Day 1</th>
<th>Postprocedure</th>
<th>Preprocedure</th>
<th>Day 5</th>
<th>Postprocedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemoglobin (g/dl)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Mean</td>
<td>11.4</td>
<td>10.0</td>
<td>10.9</td>
<td>9.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.9</td>
<td>1.06</td>
<td>0.9</td>
<td>9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>11.7</td>
<td>9.8</td>
<td>10.9</td>
<td>9.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>9.5-13.0</td>
<td>9.3-11.9</td>
<td>9.4-11.4</td>
<td>8.5-12.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total White Cell Count (×10⁹/liter)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>7.4</td>
<td>6.1</td>
<td>5.4</td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.7</td>
<td>1.3</td>
<td>1.4</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>6.9</td>
<td>5.7</td>
<td>5.4</td>
<td>5.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6.0-11.1</td>
<td>4.5-8</td>
<td>2.9-7.5</td>
<td>4.6-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymphocytes (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>28</td>
<td>29</td>
<td>34</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>11.4</td>
<td>9.3</td>
<td>8.6</td>
<td>9.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>1.9</td>
<td>1.7</td>
<td>1.9</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>16-48</td>
<td>20-48</td>
<td>25-48</td>
<td>1.8-48</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Platelets (×10⁹/liter)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>283</td>
<td>231</td>
<td>222</td>
<td>188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>70.3</td>
<td>40</td>
<td>58.7</td>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>283</td>
<td>231</td>
<td>222</td>
<td>188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>166-387</td>
<td>178-277</td>
<td>145-313</td>
<td>121-256</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mean for platelets is P = .05; none of the other differences before and after the procedure are statistically significant.

RESULTS

Donor Tolerance and Side Effects

There was no morbidity during any of the 195 procedures carried out on the marrow donors (n = 35) or the additional procedures undertaken on the volunteers (n = 15). Specifically, by using prophylactic calcium, no symptoms or signs of hypocalcaemia developed. The latter practice is based on our observations that ionizable calcium falls during cell collection while total calcium and magnesium levels are not altered (Jacobs and Wood; unpublished observations). All procedures were carried out by means of a vein-to-vein circuit, with cannulae introduced under local anaesthetic for each separate collection; these were excellently tolerated without exception.

Haematologic Changes In Vivo

In the bone marrow donors undergoing intensive white cell collections (n = 35) (Table I), the median values before and after the procedure were as follows: haemoglobin, 11.7 and 9.8 g/dl on day 1 and 10.9 and 9.1 on day 5; total white count, 6.9 and 5.7 × 10⁹/l on day 1 and 5.4 and 5.1 on day 5; lymphocytes, 1.9 and 1.7 × 10⁹/l on day 1 and 1.9 and 1.4 × 10⁹/l on day 5; platelets, 283 and 231 × 10⁹/l on day 1 and 222 and 188 × 10⁹/l on day 5.

In the volunteers (Table II) the median values immediately before and after the procedure and those in the bag of cell-rich plasma, respectively, were as follows: haemoglobin, 11.4, 10.6, and 1.8 g/dl; total white count, 5.9, 5.2, and 46.5 × 10⁹/l; lymphocytes, 30%, 30%, and 84%; platelets, 260 and 209 × 10⁹/l with a yield of 1.7 × 10¹¹.

COMMENTS

No invasive procedure is without risk, and donor safety becomes of paramount importance when this is undertaken without direct benefit to the individual. The repeated use of marrow donors for intensive collection of cells to enhance engraftment or of volunteers to provide mononuclear cells as a biological reagent for standardisation of laboratory techniques was therefore studied and shown to be safe, without discomfort to the donor, and to have no demonstrable effect on haemoglobin, white cell count, or lymphocyte numbers. Furthermore, the intensive cell collection in 35 bone donors carried out daily over a 5-day period was similarly unassociated with significant change in these measurements. In both groups there was a short-lived fall in platelet count, which was not associated with any bleeding disorders and which had returned to baseline level within 24 hours of collection. However, in the donors undergoing the more intensive
TABLE II. Haematologic Changes In Vivo—Normal Volunteers†

<table>
<thead>
<tr>
<th>Haemoglobin (g/dl)</th>
<th>Preprocedure</th>
<th>Postprocedure</th>
<th>Total bag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>11.5</td>
<td>10.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.7</td>
<td>1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Median</td>
<td>11.4</td>
<td>10.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Range</td>
<td>9.0–17.4</td>
<td>7.2–17.0</td>
<td>.8–2.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total White Cell Count (×10^9/liter)</th>
<th>Preprocedure</th>
<th>Postprocedure</th>
<th>Total bag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>6.0</td>
<td>5.5</td>
<td>45.9</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.4</td>
<td>1.0</td>
<td>14.2</td>
</tr>
<tr>
<td>Median</td>
<td>5.9</td>
<td>5.2</td>
<td>46.5</td>
</tr>
<tr>
<td>Range</td>
<td>2.9–11.1</td>
<td>4.2–8.0</td>
<td>23.6–80.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lymphocytes (%)</th>
<th>Preprocedure</th>
<th>Postprocedure</th>
<th>Total bag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>31</td>
<td>32</td>
<td>80</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>10.2</td>
<td>0.2</td>
<td>14.9</td>
</tr>
<tr>
<td>Median</td>
<td>30</td>
<td>30</td>
<td>84</td>
</tr>
<tr>
<td>Range</td>
<td>10–48</td>
<td>16–48</td>
<td>50–100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Platelets (×10^9/liter)</th>
<th>Preprocedure</th>
<th>Postprocedure</th>
<th>Total bag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>245</td>
<td>212</td>
<td>1.8*</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>60</td>
<td>50</td>
<td>0.6*</td>
</tr>
<tr>
<td>Median</td>
<td>250</td>
<td>209</td>
<td>1.7*</td>
</tr>
<tr>
<td>Range</td>
<td>112–387</td>
<td>127–306</td>
<td>0.9–3.2*</td>
</tr>
</tbody>
</table>

†None of the differences pre- or postprocedure is statistically significant.

The units for the platelet yield in the total bag are ×10^9/liter and not ×10^9/liter.

schedule, the difference between the initial preprocedure and the final postprocedure counts just reached a statistical significance (P = .05).

Although the procedures are strictly isovolaemic, the small and statistically nonsignificant reductions in haematologic values are thought to reflect minimal dilution, and, since they had all returned to baseline levels within 24 hours, emphasize that no cellular deficiency occurs as a result of the apheresis procedure.

These same observations suggest that if large quantities of mononuclear cells must be removed in an attempt to modulate immunologically mediated disease, then much more intensive schedules would be needed and should be seen as adjunctive to immunsuppressive therapy [14,15].

It is concluded that mononuclear cell separation and collection employing continuous-flow centrifugation is a safe and convenient way of cell harvesting in selected clinical situations and also as a source of biological reagents; large numbers of cells can be harvested safely and without significant depletion so that the haematologic status of the donor is not compromised.

ACKNOWLEDGMENTS

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REFERENCES


In Vitro Study of Immunologic Changes in Long-Term Cytapheresis Donors

Clive R.B. Prior, Patrick J. Coghlan, Jane M. Hall, and Peter Jacobs

 Natal Blood Transfusion Service, Durban (C.R.B.P.), Western Province Blood Transfusion Service, Cape Town (P.J.C.), Institute for Biostatistics of the South African Medical Research Council, Tygerberg (J.M.H.), and University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Observatory, Cape (P.J.), South Africa

Several in vitro measurements of immune function were examined retrospectively in a population of active long-term cytapheresis donors (group I; n = 50) and the results were compared to age- and sex-matched controls (group II; n = 50) who had donated only whole blood. In group I, significantly different mean absolute lymphocyte counts (P < .0025), total T-cells (P = .0026) and T-helper cells (P < .0001), and helper-to-suppressor ratios (P = .0279) were present. No differences were noted between the two groups for peripheral blood mean B-cell count, T-suppressor numbers, lymphocyte responsiveness to mitogens or alloantigens, and serum immunoglobulin level. The reduced mean absolute lymphocyte count in group I was due to the reduction in T-helper cell numbers and accounted for the imbalance in the helper-to-suppressor ratio. These disturbances are currently unexplained and, while no clinical consequences have so far become evident, there is a need to continuously monitor the immunologic status of cytapheresis donors. It is also important to determine whether reversal of the defects occurs and, if so, over what time interval.

Key words: cytapheresis, lymphocyte subsets, mitogen response

INTRODUCTION

Concern has been expressed about possible health risks to long-term cytapheresis donors [1–7], based on the observation that many otherwise healthy individuals have become lymphocytopenic, probably as a result of chronic loss of this cell population. Any risks attributable to this phenomenon remain unknown, but potential hazards of changes in immune competence include infections, malignancy, and autoimmune disorders [5]. In this regard, reliable information on alterations in circulating blood lymphocyte subpopulations and immune function in normal healthy donors undergoing repeated mechanical cytapheresis is limited [4,7,8].

Guidelines issued in August 1981 by the Food and Drug Administration (FDA), Office of Biologics [9] for the collection of platelets by cytapheresis techniques do not refer to lymphocyte loss. In an update [10], based largely on the availability of new separators, where loss is approximately 1 × 10^7 lymphocytes per procedure, 24 platelet collections per year are allowed. In addition, an expert panel published advice on how to prevent the development of moderately severe lymphocytopenia, defined as <1 × 10^9/L, in the apheresis donor [5], but conceded that insufficient quantitative data was available for circulating lymphocyte subpopulations to influence decisions regarding blood donations.

Since donors make an important contribution to the community, it is the responsibility of those running apheresis programmes to ensure that the health and safety of participants receive constant attention and that these be balanced against the need to procure products of high quality.

MATERIALS AND METHODS

Trial Design

In the years 1984 and 1985 a comparative study was undertaken between a sample of members of a long-term cytapheresis panel and a reference group, matched for age, race, and sex, who had donated only whole blood, to identify the effects of apheresis on absolute blood lymphocyte count and subpopulations, response to stimulation with mitogen or alloantigen, and serum immunoglobulin level.

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This work was based on a thesis by Clive R.B. Prior submitted in partial fulfillment of the degree of Master of Medicine in Pathology (Haematology), Department of Haematology, University of Cape Town, Cape, South Africa.

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globulin levels. None of the individuals was taking medication known to influence lymphocyte numbers.

**Subjects**

Healthy volunteers were randomly selected, each having fulfilled the requirements of the American Association of Blood Bank Standards [11] applicable to whole blood or cytapheresis donation. The study was approved by the University of Cape Town and Groote Schuur Hospital Ethics and Research Committee; informed consent was obtained from all participants. Exclusions were on the basis of recent infection or other illness.

Group I (n = 50) consisted of 48 males and 2 females, with a mean age of 37 years (range 25–51). All had previously donated whole blood (mean 26; range 3–81) and were now exclusively on a cytapheresis programme, having undergone a minimum of 25 procedures each (range 25–102) over a 3 to 9 year period, with a maximum of 12 procedures a year. A total of 1,949 aphereses were performed: 1,743 (89.5%) to harvest platelets and 206 (10.5%) for granulocyte collection. The latter were restricted to a maximum of two per year (mean 5; range 0–13).

Controls consisted of regular whole blood donors, individually matched for age and sex (group II; n = 50), who had given a comparable number of whole blood donations to the study group (mean 29; range 11–82). None of these individuals had undergone a cytapheresis procedure.

**Cytapheresis Protocols**

The centrifugal blood cell separators (CBCS) utilised were models 30 and V50 (Hemometrics Corporation, Braintree, MA) or the CS3000 (Fenwall, Deerfield, IL). The operating conditions for thrombocytepheresis procedures for each machine are set out in Table 1. The surge pump modification of the Hemometrics V50 was not in operation during this study period. Average cell recoveries were calculated from routine quality assurance data of 25 consecutive procedures with each machine. Leucocytepheresis donors were premedicated, when possible, with 60 mg prednisone orally 8 hours before donation [12].

**Donor Samples**

In order to minimise the effects of diurnal variation, recent cytapheresis, and the administration of starch or steroid, all donor samples were collected between 08h00 and 09h30 at least 3 weeks after the last cytapheresis procedure.

**Haematological and Biochemical Investigations**

Haemoglobin, packed cell volume, total leucocyte and platelet count were determined using the Coulter

<table>
<thead>
<tr>
<th>TABLE 1. Thrombocytepheresis Data*</th>
</tr>
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<tbody>
<tr>
<td>Hemometrics</td>
</tr>
<tr>
<td>Whole blood processed (L)</td>
</tr>
<tr>
<td>Procedure time (minutes)</td>
</tr>
<tr>
<td>Product volume (mL)</td>
</tr>
<tr>
<td>Platelet recovery × 10^11</td>
</tr>
<tr>
<td>Leucocyte recovery × 10^9</td>
</tr>
<tr>
<td>Red cell contamination (mL)</td>
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</tbody>
</table>

*Mean of 25 procedures with each centrifugal blood cell separator (CBCS).

Anticoagulant ACD-B in a ratio of 1:8.

PROM 6 protocol modified for optimum platelet recovery; anticoagulant ACD-A in a ratio of 1:13.

Counter, model S-Plus II Automated Electronic Hematology Analyser (Coulter Electronics, Hialeah, FL) and the white blood cell differential using the Hematrak, model 480, (Geometric Data, Wayne, PA) counting 400 leucocytes.

Serum ferritin values were determined using a radioimmunoassay kit (Amersham International, UK). Serum IgG, IgA, and IgM were quantitated by nephelometric immuno-analysis, using the Behring Laser Nephelometer (Behringwerke AG, Marburg, West Germany) according to the manufacturer's instructions. Anti-HIV antibody assays were performed using an Abbott EIA kit (Abbott Diagnostic Products, Wiesbaden-Kelkenheim, West Germany) on all donors showing abnormal OKT4 to OKT8 ratios.

**Lymphocyte Preparation**

Donor peripheral blood lymphocytes were isolated from heparinised blood specimens by density gradient centrifugation (18–20°C at 400g) in Lymphoprep (Nyegaard & Co., Oslo, Norway) (sodium metrizoate/Ficoll solution; 1.077 g/mL) [13]. After washing three times in Hanks' balanced salt solution the lymphocytes were suspended in 5% foetal calf serum (Gibco, Grand Island, NY), in Hanks'†/HEPES buffer, and the cell count was adjusted to 4–6 × 10^9/L. Ninety-five percent of all cells were viable by trypan blue exclusion.

**Quantitation of T- and B-Lymphocytes**

Circulating B-lymphocytes were enumerated, using a direct immunofluorescent technique [14] for surface membrane immunoglobulin (SmIg). Fluorescein isothiocyanate (FITC) conjugated (F(ab')2 goat antihuman immunoglobulin (polyvalent) (Kallestad Laboratories, Austin, TX) was used to avoid the problem of conjugate binding to Fc receptors. Wet mounts of the cell suspensions were examined under a Nikon episcopic-fluorescent microscope at 400 × , the fluorescent (SmIg
positive) cells being expressed as a percentage of the total number of lymphocytes visualised.

Absolute numbers were then calculated, using the peripheral blood lymphocyte count.

T-cells were enumerated by their ability to form stable E-rosettes, using 2-aminoethylisothiouronium (AET) bromide-treated sheep erythrocytes [15].

Quantitation of T-Lymphocyte Subsets

T-lymphocyte subsets were identified and counted, using Orthomune (Ortho Diagnostic Systems, Inc., Raritan, NJ) murine monoclonal antibodies in an indirect immunofluorescence test, according to the manufacturer’s instructions. The monoclonal antibody panel employed consisted of OKT3, which identifies human T-lymphocytes (Pan-T) expressing the 19,000 dalton cell surface antigen, OKT4, expressing specificity for the 60,000 dalton cell surface antigen, primarily with inducer and helper function, and OKT8, recognising 31,000 dalton cell surface antigen, having suppressor and cytotoxic functions. Absolute numbers of T-cell subsets were calculated from the percentage of lymphocytes exhibiting fluorescence and the peripheral blood absolute lymphocyte count. The OKT4 to OKT8 ratio was calculated for each individual in the study.

Stratification by Immunophenotype

Results from the donors in each group were stratified into absolute lymphocyte counts <1.5 × 10⁹/L; OKT4 to OKT8 ratio <1.5; OKT4 to OKT8 ratio <1.0; absolute lymphocyte count <1.5 and OKT4 to OKT8 ratio <1.5; absolute lymphocyte count <1.5 and OKT4 to OKT8 ratio <1.0.

In Vitro Lymphocyte Proliferative Responses

In an assay standardised for cell numbers and concentration of mitogen, the incorporation of tritiated thymidine was compared between lymphocytes from donor and control and the results of the two groups expressed as a ratio [16]. Parallel studies using alloantigen were defined in the one-way mixed lymphocyte reaction [17], in which lymphocytes separated from a non-donor and stored in liquid nitrogen functioned as stimulators following inactivation by exposure to 25 μg of mitomycin C at 37°C for 30 minutes.

Statistical Methods of Analysis

The sample size permitted the use of the paired t-test to compare the cytapheresis donor group to the whole blood donor group. McNemar’s exact test for matched-pair data was used to examine the proportions when variables were categorised. The 5% significant level was used throughout to test the hypotheses.

RESULTS

Sample statistics for the variables were measured, and the results of the comparisons of the cytapheresis donors (Group I; n = 50) to the controls (group II; n = 50) are shown in Tables II and III. Technical errors in assay procedure account for sample sizes of less than 50.

Haematological and Biochemical Measurements

The mean haemoglobin level was statistically different (P = .0015), being higher in group I, with no anaemic individuals and only two in this group having serum ferritin levels less than 20 ng/mL. Although there was no difference in the mean serum iron and ferritin levels between group I and group II, three whole blood donors (group II) were iron deficient and a further 6 had borderline values.

The mean platelet counts of the two groups were comparable and in both instances within the normal range.

There was no difference in the mean total leucocyte counts between the two groups. However, the mean absolute lymphocyte count in group I was lower than that of group II and statistically different (P = .0025) (Table II). In addition, the proportion of lymphocytopenic donors, defined by absolute counts <1.5 × 10⁹/L, differed between the two matched groups (P = .0170) (Table IV) and was higher in the cytapheresis group (P = .0273).

The mean absolute lymphocyte loss from volunteers in group I, using the Hemotek cell separator, was calculated to be 0.5 × 10¹⁰ per procedure, with a corresponding value of 0.02 × 10¹⁰ for the Fenwall CS3000 (Table I). Surprisingly, 17/50 (34%) of the whole blood controls (group II) were found to have an absolute lymphocyte count <1.5 × 10⁹/L (Table IV). No significant difference in the mean values for any of the serum immunoglobulins was demonstrable between the two groups (Table II). All individuals in group I with abnormal OKT4 to OKT8 ratios were negative for HIV-antibody.

In the years the study was conducted anti-HIV testing was not universally established, although donors with a history for risk of this retroviral infection were, as far as possible, excluded. It nevertheless remains theoretically possible, although unlikely, that infected individuals could have been included in either group I or group II. However, in the ensuing years, when HIV testing has been routine, the very low incidence rate, for practical purposes, excludes this possibility.

Lymphocyte Studies

The results in our laboratory for total lymphocyte numbers and subsets was comparable between the control patients in group II and random normal subjects drawn from a population of similar age, where no significant differences were demonstrable between men and
women. It is recognised that blood samples were collected at a time when absolute lymphocyte counts would be at their lowest and the non-donor population was studied at the same time; under these circumstances, diurnal variation for the subsets was not carried out.

The mean percentage of B-cells, defined by SmIg positivity, differed between the two groups (P = .0002) and was higher in group I, whereas the mean absolute values for the two groups were not different. Conversely, the T-cell data showed no difference in the mean T-cell percentage between group I and group II (P = .1456). In contrast, when absolute values, defined by the Pan-T monoclonal antibody (OKT3) are considered, a lower value is evident in group I (P = .0026). Further investigation of the T-cell subsets, each expressed as a percentage of the absolute lymphocyte counts, showed that in group I the mean percentage for the helper cells, identified by the OKT4 antibody, was associated with a higher mean percentage of suppressor cells, recognised by OKT8. A comparison of group I to group II demonstrated a significant difference for OKT4% (P = .0006) and OKT8% (P = .0001).

Examination of the mean absolute counts, however, showed a statistically significant difference (P < .0001) and revealed a marked reduction in the OKT4 positive cell population in group I, but no difference in the number of OKT8 positive cells in comparison to the controls. This imbalance in absolute numbers is reflected in the
TABLE IV. Analysis of Lymphocyte Count and Immunophenotyping

<table>
<thead>
<tr>
<th>Categories of variables</th>
<th>Proportion of cytopheresis donors (group I)</th>
<th>Proportion of whole blood donors (group II)</th>
<th>McNemar's exact probability test for matched pairs (two-tailed)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute lymphocyte count &lt;1.5 x 10^9/L</td>
<td>29/50</td>
<td>17/50</td>
<td>.0170</td>
</tr>
<tr>
<td>OKT4:OKT8 (Helper:suppressor) ratio &lt;1.5</td>
<td>28/50</td>
<td>8/50</td>
<td>.0002</td>
</tr>
<tr>
<td>OKT4:OKT8 (Helper:suppressor) ratio &lt;1.0</td>
<td>10/50</td>
<td>2/50</td>
<td>.0220</td>
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<tr>
<td>Absolute lymphocyte count &lt;1.5 x 10^9/L + OKT4:OKT8 ratio &lt;1.5</td>
<td>16/50</td>
<td>1/50</td>
<td>.0002</td>
</tr>
<tr>
<td>Absolute lymphocyte count &lt;1.5 x 10^9/L + OKT4:OKT8 ratio &lt;1.0</td>
<td>5/50</td>
<td>1/50</td>
<td>.2189</td>
</tr>
</tbody>
</table>

*There were 50 individuals in each of the two groups. Statistical significance using the paired t test was defined as values less than P = .05.
†Yates corrected chi-squared test used (see text).

The proportion of individuals in each of the two groups with a reduced OKT4 to OKT8 ratio (<1.5) or when this was reversed (<1.0) is set out in Table IV. Both categories showed a marked larger proportion of donors from group II. Of greater importance is the preponderance of individuals from group I who were both lymphocytopenic and had an OKT4 to OKT8 ratio of <1.5 (16/50 versus 1/50; P = .0002) (Table IV); this cut-off value, defining lymphocytopenia and clinical significance for ratio reversals, was previously defined in our laboratory. There was, however, no difference in the proportion of donors with a combination of lymphocytopenia and reversal in the OKT4 to OKT8 ratio, although the number is small.

The results of the in vitro lymphocyte function tests were not different between the two groups when lymphocyte responsiveness was tested to mitogens or by stimulation with alloantigen in the one-way mixed lymphocyte reaction (MLR) (Table III). It is notable, however, that on exposure to phytohaemagglutinin, which is predominantly a T-cell mitogen, a difference (P = .0279) (Table III) was found, whilst reactivity amongst individuals from group I was lower.

DISCUSSION

The finding of iron deficiency in the control group is consistent with the significant erythrocyte loss that accompanies regular whole blood donation and leads to depletion of body stores [18-20]. In contrast, apheresis procedures are associated with minimal red cell contamination of the harvested product and they are unlikely to result in changes in serum iron or ferritin levels [20]; an observation confirmed in results from our long-term cytopheresis donors (Jacobs & Wood; unpublished observation).

All the volunteers in group I sustained large platelet losses over a period of 3 to 9 years, yet each maintained a peripheral count within the normal range. These results are in keeping with the previously reported studies [4,21-23] and reflect accelerated megakaryocytopenia in response to increased peripheral demand.

Although substantial numbers of granulocytes are removed during cytopheresis, no donor in this study was found to be neutropenic. The maintenance of circulating levels is attributed to rapid cellular replacement [24] from the marrow granulocyte pool and also to intravascular demargination that follows prednisone premedication. The single dose of steroid, given once with each procedure, is unlikely to have, in any way, affected these results. Furthermore, it has been stated that these cells cannot be removed by any present-day technique at a rate sufficient to result in circulating levels [21].

Lymphocyte depletion, however, poses more of a problem to the long-term cytopheresis donor. The mean absolute loss during thrombocytopheresis, using the Hemonetics Model 30 CBCS, was calculated to be 0.5 x 10^10 on each occasion. It follows that 12 such procedures a year result in 15 times more lymphocytes being lost than when a person makes four donations annually. The estimated total body mass of lymphocytes is at least 1 x 10^12 cells [24]. Therefore, the individuals in group I could have lost the equivalent of 12-50% of their total lymphocyte pool over a 3 to 9 year period. This postulate is consistent with the significantly reduced circulating lymphocyte counts in long-term cytopheresis donors.
when compared to the control group, and confirms two previous reports [3,4,7,8]. Thus, we are obliged to attribute the changes exclusively to the apheresis procedures and it appears that reductions are time-related and may reflect the number of procedures undertaken, but insufficient data are available to estimate statistical value to this observation. Particularly noteworthy is the fact that counts do not return to higher or even normal levels on stopping the cytapheresis. Currently unexplained is the observation that one-third of the individuals in group II were also found to be lymphocytopenic. Attempts to determine whether these were poor controls or whether the accepted lower limits for lymphocyte counts may be inaccurate for this population of men studied in the mornings failed to show any bias and accordingly this is the subject of further study.

The reduced absolute lymphocyte counts in group I appear to be due to a highly significant decrease in the numbers of circulating T-helper cells, resulting in an imbalance of the OKT4 to OKT8 ratio in many individuals. It has, however, not been possible to confirm two other reports [4,7,8] of a significant reduction of suppressor or cytotoxic subsets, with mean absolute counts of OKT8 positive cells being comparable in groups I and II. An alternative explanation, however, is that the latter population is replenished more rapidly than the OKT4 population [8]. No difference was found between mean absolute B-cell counts in our two groups, thereby confirming two previous reports [4,8]. These findings are in contrast to reports of significant decrease in B-lymphocyte levels in cytapheresis donors [2,3]. Furthermore, our findings of comparable mean serum immunoglobulin levels between groups I and II differ from the significant decrease in immunoglobulin levels previously reported [3,8] in individuals undergoing platelet apheresis.

Knowledge is incomplete on the way in which lymphocytes are produced and peripheral blood levels are regulated [25], but information on T-cell kinetics, including their longevity [26], reduced functional activity associated with increasing age [27,28], and the presence of specific regulatory subsets raises the possibility that normal immunologic competence may be altered by their recurring loss. Thus, drainage of 5.7 x 10^10 lymphocytes from the thoracic duct, 90% of which are T-cells, impairs their functional activity, including allograft rejection, substantially diminishing primary antibody response to bacterial antigens and producing long-lasting depression of delayed hypersensitivity [29,30].

In the present study, in vitro lymphocyte function tests demonstrated significantly lower responsiveness to the T-cell mitogen, phytohaemaggulutinin, in comparison to the whole blood donor controls. In this regard, the established interaction between the mononuclear cells in these in vitro culture systems [16], giving rise to the final response to incorporation of tritiated thymidine, may be dampened by the reduced proportion of T-helper cells that we have shown to be present in the long-term cytapheresis donors.

Interpretation of our data is consistent with protracted loss of T-cells in group I, analogous to thoracic duct drainage; although in these situations the patients were not normal, and the persistence of this defect suggests that replenishment may not be as efficient as might have been anticipated. It followed that restoration of in vivo immune function, reflected in reconstitution of in vitro tests, will require correction of T-cell loss, and definition of this sequence of events will necessitate further long-term observation.

It is difficult to explain the discrepancy in reconstitution of OKT4 and OKT8 subpopulations by apheresis procedures. Differential removal of these two populations or inhibition of T-helper cell production by the procedure itself seems unlikely. Thus, if T-lymphocytes are harvested in the same proportion as peripheral blood levels, then with a normal helper-to-suppressor ratio it would be anticipated that twice as many OKT4 as OKT8 cells would be lost. Assuming that the peripheral blood T-lymphocyte subset ratios faithfully reflect their distribution throughout the total body pool, estimated at being between 30 and 40 x 10^10 cells [1], then a significant reduction in the peripheral T-helper cells in the cytapheresis donors is cause for concern.

The results of the present study suggest that recommendations based solely on the absolute peripheral blood lymphocyte count [5] are inadequate because they do not reflect the differential subset loss. Thirteen, or 26%, of the long-term cytapheresis donors with normal absolute lymphocyte counts had reduced helper-to-suppressor ratio of <1.5, and of these five had reversal, with ratios <1.0. It would seem appropriate, therefore, that on entry to a cytapheresis programme absolute as well as lymphocyte subsets be documented. Furthermore, the current observations would suggest that these measurements should be monitored on a serial basis, and it remains to be determined which interval would be most appropriate. Although it is accepted that lymphocyte sparing techniques should be used, no prospective study has, to date, been published to establish safety in terms of total number or frequency of cytapheresis procedures, and such information appears to be urgently needed.

Finally, there is no information available to exclude the possibility that subtle or occult immune dysfunction may have been created in some long-term cytapheresis donors and that the consequences would become manifest only under conditions of stress. Our data, based on in vitro tests, demonstrate that immune balance already existed in many of these individuals at the time they
were studied, but the cause for this is not clear. Careful evaluation of the various laboratory values being analyzed suggests that those having statistical significance have not been over-interpreted. To date, no in vivo changes have emerged in altered immune function and neither have the changes had any clinical consequences.

The failure to demonstrate similar defects in the control population would appear to exclude prior blood transfusion as contributory and incriminate the apheresis procedures in the generation of this immunologic defect. Accordingly, we support the recommendation [5] that a Registry be established for donors on cytopheresis programs, particularly where lymphocyte abnormalities have already been documented, and long-term surveillance be maintained. It should be noted that these data cannot be extrapolated to the Hemonetics V50 surge or the third generation centrifugal separators, exemplified by the COBE Spectra; it is entirely possible that lymphocyte depletion using the more modern instruments may approximate those found with whole blood donation, but this point has not, to our knowledge, been established.

An additional benefit of such monitoring would be an ability to document an increase in the incidence of infection, malignant disease, autoimmunity or other clinically important conditions arising in the study population. It would be appropriate that such individuals be excluded from further donation.

ACKNOWLEDGMENTS

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REFERENCES


Immunologic Changes in Cytopheresis Donors 75


The effect of peptide stimulation on haematopoietic stem cell mobilisation including engraftment characteristics and a note on donor side effects

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Abstract

Aplasia or irreversible bone marrow failure and a variety of haematologic malignancies, as well as an increasing number of solid tumours, currently include various forms of marrow or equivalent transplantation in routine management. In both allogeneic and autologous procedures stable recipient immunohaematopoietic reconstitution depends upon infusing the requisite population harvested at a precise time following commencement of a stimulatory peptide. In a first step this prospective study documented the safety of apheresis, defined side effects and enumerated mononuclear, CD34+ and CD3+ cells obtained. In the second stage delivery of the graft, characterised in this way and with the additional measurement of in vitro growth in clonogenic assay, to the suitably conditioned patient was correlated with recovery of neutrophil and platelet numbers appearing in the circulation. In a third and ongoing analysis the influence of passenger T-lymphocytes is being evaluated for impact on infection and a potential anti-tumour effect. The conclusion is that this technology is reliable, has a high degree of patient acceptability without untoward complications, and that local results correspond to international experience thereby providing an important and relevant measure of quality control.

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1. Introduction

Escalation of chemotherapy, often combined with irradiation, increasingly reaches levels that require support by infusion of haematopoietic progenitors. This intervention is accepted as integral to the standard care of those with irreversible
marrow failure, many haematologic neoplasms and an expanding array of selected solid tumours. Obtaining such donations from the peripheral blood, using apheresis technology, has superseded the older practice of multiple bone marrow aspirations as the source of the graft. Successful outcome is governed by three broad variables. The first of these is patient-based and includes disease category whilst current status, coupled with prior therapy, may influence efficient mobilisation of an autograft. The second, also in the recipient, is residual integrity of the haematopoietic inductive microenvironment known to be essential in supporting recovery of blood formation and immune function. This latter tissue may have been damaged by conditioning or preparative regimens of which most are still myeloablative. Thirdly is the composition of the donation reflected in total lymphocytes and monocytes as well as CD34+ precursors measured by flow cytometry whilst repopulating potential is quantitated in colony formation using calibrated in vitro culture techniques. The interplay of these factors combine to determine the efficiency with which neutrophils and platelets reappear in the circulation. In the longer-term recovery of immunity becomes the dominant influence on a variety of infections and, ultimately, disease-free survival.

It is an ethical obligation that collection procedures are carried out with attention to donor safety and recognition that side effects may arise from the administration of molecules such as GM or G-CSF and this is particularly important in volunteer participants. Prior discussion should therefore provide reassurance and balanced information about the possibility of musculo skeletal discomfort that may be associated with self-administration of this agent. Additionally the procedure needs to be outlined so that there is familiarisation with the instrument and the various manoeuvres that take place during harvesting while the continuous presence of an experienced nurse is integral to donors peace of mind and safe management.

This ongoing trial monitors each consecutive apheresis and contrasts allogeneic and autologous experience constantly comparing results to publications [1,2] as part of safety evaluation in this department.

2. Patients

Details of the entire procedure were explained including technicalities of venous cannulation, the need and method required for mobilisation into the circulation as well as the voluntary nature of the participation. Written informed consent was obtained in each instance. Viral screens were carried out and individuals medically examined for suitability to donate. Neupogen (Roche Products SA) was administered at a dose of 300 μg subcutaneously starting on the Friday as a matter of convenience and this was designated as day 0. Where possible a vein-to-vein technique was used. All patients were interviewed on a daily basis and side effects documented through a minimum of one week [3].

3. Methods

Peripheral blood specimens were collected into EDTA as a baseline and subsequently on days 3, 4, 5 and 7. Full blood and differential counts was performed on the Cell-Dyn 1700 Haematology analyser [4] and 1000 cell differential noted on each occasion with calculation of absolute leucocyte numbers [5].

Flow cytometry was performed on each sample to quantitate CD3 and CD19 populations [6]. Two different techniques were used for CD34 quantitation but there was no overlap of methodology during any particular collection.

The ISHAGE [7] method required 100 μl of patient’s blood to be incubated with 10 μl of CD34 and CD45 antibodies. After lysing and washing, dual expression of the two surface markers was detected using direct immunofluorescence and analysed using the recommended gating strategy.

In the ProCOUNT (Becton Dickinson) [8] approach, a two-tube assay was performed by staining the sample with CD34 and Control reagent in individual TruCOUNT tubes. When using an appropriate reaction mixture fluorochrome-labelled antibodies bound specifically to surface membrane antigens while nucleic acid dye stained the DNA and RNA. The lyophilised pellet is then dissolved, releasing a known number of fluorescent beads in the Control, and so correcting for the
amount of non-specific antibody binding. FACS
lyse solution is added to destroy erythrocytes be-
fore analysis on the FACSscan flow cytometer.
During counting the absolute number of CD34+
events is determined by correcting these for signals
generated by the beads and then multiplying this
by the concentration of the latter.

Individual collections were standardised to day 4
based upon a previous study (Jacobs and Wood,
unpublished) as well as recommended guidelines
[9]. The Cobe Spectra instrument was used to pro-
cess between 3 and 5 times the estimated blood
volume with anticoagulant being acid citrate dext-
rose-A solution (ACD-Baxter SA). Calcium
replacement was as 10% gluconate (Baxter SA)
at a rate of 6ml/h with appropriate increases for
the development of symptomatic hypocalcaemia.
The volume and contents of each harvest were de-
finite in terms of mononuclears, CD34+ numbers
as well as colony formation in clonogenic assay
with CD3+ positivity also specified for allografts
where measurements were carried out prior to
adding the opsonic Campath 1 H monoclonal anti-
body [10]. Autografts were cryopreserved and

Reinfusion took place over 1 h and was pre-
ced by premedication with 100 mg of hydrocortisone,
12.5 mg of phenergan and 2 paracetamol
tablets orally. Thereafter the reappearance of
granulocytes and platelets in the peripheral blood
was noted and this pattern correlated with the cel-
lar content of the graft [12]. The T-lymphocyte
load is to be evaluated as a predictor for the in-
cidence of post-transplant infections [13] and the
potential development of acute or chronic graft-
versus-host disease [14].

4. Results

These are given in median and range as well as
mean with standard deviation.

4.1. Donor side effects

None were documented in nine procedures and
in the remainder were minor. Lower back pain
(n = 21) was associated with injections as was
headache (n = 16) and transient heaviness on the
chest (n = 6). A number of these occurred together
in the same patient. Mild citrate toxicity (n = 5)
was immediately reversed by increasing the rate
of calcium replacement.

4.2. Collection characteristics

The volume in the bag was 474 (222–645) or 388
(±73.9) mL. A single large volume apheresis was
adequate in the majority (n = 33). A second pro-
dure was needed in 4 and a third procedure in 1
but, apart from the increased amount of plasma
recovered, no adverse effects occurred.

In the allografts (n = 27) the peak in both
mononuclear cells (Fig. 1(a)) and the circulating
CD34+ population (Fig. 1(b)) occurred on day 4
in most patients. Mononuclears recovered were
6.6 (6.6–12.5) or 8.0 (±1.3) × 10^9/kg; CD3+ 4.8
(2.1–7.5) or 0.55 (±1.13) × 10^9/kg; CD34+ 3.9
(2.7–11) or 5.3 (±1.7) × 10^9/kg and GM-CFUc
1.0 (1.0–31.1) and 6.8 (±6.34) × 10^4/kg (Table 1).

In the autografts (n = 15) a different pattern
emerged for both mononuclear cells (Fig. 2(a))
and the CD34+ population (Fig. 2(b)) where a bi-
modal distribution was seen in the peripheral cir-
culation with a significant number of patients
having a second peak on day 7. Mononuclears
recovered were 7.5 (6.0–12.13) or 8.2 (±2.0) × 10^9/kg; CD34 1.4 (0.89–22.6) or 3.3 (±5.5) × 10^9/kg
and GM-CFUc 5.1 (1.5–16) or 7.4 (±5.0) × 10^4/kg (Table 2).

4.3. Recipient outcome

This is surprisingly comparable between the two
procedures.

In the allografts following infusion (n = 27), the
time to reach 0.5 × 10^9/L neutrophils was 12 (7–15)
or 11.5 (±1.6) and 1.0 × 10^9/L 12 (8–31) or 13
(±4.4). The corresponding figures for platelets to
reach 20 × 10^9/L was 17.5 (3–62) or 20 (±15.39),
50 × 10^9/L was 17.5 (6–77) or 25.7 (±18.87) and
100 × 10^9/L was 62 (10–100+) or 46 (±32.9).

In the autografts following infusion (n = 8), the
time to reach 0.5 × 10^9/L neutrophils was 13 (12–
22) or 14 (3.4) and 1.0 × 10^9/L 13 (12–22) or 14
(3.3). The corresponding figures for platelets to
reach $20 \times 10^9$/L was 57.5 (15–+100) or 58 (37.5), $50 \times 10^9$/L was 59.5 (19–+100) or 60.8 (38) and $100 \times 10^9$/L was 60.5 (21–100+) or 72 (34.7).

5. Discussion

Nowhere in medicine is the role of the professional nurse, as an integral and often central member of the multidisciplinary team, more evident than in routine management programmes that typically include administration of chemotherapy which becomes potentially hazardous as doses are escalated to myelosuppressive levels typically needing haematopoietic stem cell transplantation [15,16]. The latter procedures continue to undergo refinement and one of the most striking is a change from the older technique of harvesting bone marrow from the sternum and ilium [17] to its replacement by recovery of the same population from the peripheral blood using apheresis technology [18].

Although well-established two broad but overlapping areas of responsibility require constant attention to ensure that patient safety remains paramount in the delivery of health care. Firstly, while these observations apply to all donations,
they assume particular importance when siblings are used and even more so in matched unrelated volunteer donor programmes [19]. Thus ethical principles dictate a thorough familiarity with technical details, the availability of modern, safe and regularly tested equipment with experienced apheresis nurses carrying out each harvest in an atmosphere characterised by quiet efficiency and where patient fears are allayed by careful prior explanation of each step in the collection. To this must be added the anticipation of side effects where it is mandatory to balance appropriate information with objectivity on the one hand and administration of the least amount of the stimulatory peptide commensurate with obtaining optimum results on the other [20]. Secondly, and in context, it is crucial that the biological product obtained has an adequate margin of safety in terms of number and function of progenitors to ensure subsequent reestablishment of both blood formation and immunologic integrity [21,22].

Early experience with this methodology centred on the recovery of granulocytes for managing neu-
tropicen sepsis but the unstimulated yields were

<table>
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<tr>
<th>Number and diagnosis</th>
<th>Mononuclear cells (x10^9/kg)</th>
<th>CD3 (x10^9/kg)</th>
<th>CD34+ (x10^9/kg)</th>
<th>GM-CFUe (x10^5/kg)</th>
<th>Days to peripheral blood response</th>
<th>Neutrophils (x10^9/L)</th>
<th>Platelet (x10^9/L)</th>
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<td>23. DH</td>
<td>7.4</td>
<td>5.6</td>
<td>2.8</td>
<td>2.3</td>
<td></td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>24. KX</td>
<td>8.9</td>
<td>6.9</td>
<td>3.1</td>
<td>8.6</td>
<td></td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>25. MG</td>
<td>7.9</td>
<td>2.1</td>
<td>8.7</td>
<td>31.1</td>
<td></td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td>26. LL</td>
<td>8.3</td>
<td>6.3</td>
<td>6.1</td>
<td>13.2</td>
<td></td>
<td>9</td>
<td>54</td>
</tr>
<tr>
<td>27. KL</td>
<td>9.8</td>
<td>5.4</td>
<td>1.4</td>
<td>4.7</td>
<td></td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Median</td>
<td>6.6</td>
<td>476</td>
<td>3.9</td>
<td>1.0</td>
<td>12</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>6.6-12.5</td>
<td>206-750</td>
<td>2.7-11</td>
<td>1.0-31.1</td>
<td>7-15</td>
<td>3-62</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>8.0</td>
<td>54.8</td>
<td>5.3</td>
<td>6.8</td>
<td>11.5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>1.3</td>
<td>112.7</td>
<td>1.7</td>
<td>6.34</td>
<td>4.4</td>
<td>15.39</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2. Autologous donors. Both cell populations follow a similar pattern but a bimodal distribution is noted in the CD34 cells with a second peak on day 7. This observation does not appear to have been previously documented and may reflect changes known to occur in the haematopoietic micro inductive environment and is not necessarily a consequence of prior myeloablative therapy and bone marrow transplantation [59].

relatively small and, except in children, of limited value. With the availability of highly purified molecules, known to raise white cell count, this aspect of transfusion therapy is again assuming clinical importance [23]. Thus, on the basis of the older model rests the scope of harvesting intramedullary progenitors during their passage through the circulation by means of apheresis technology [24]. Initial observations showed a marked increase in recovery following chemotherapy [25] but the use of cytotoxic drugs in otherwise healthy donors is inappropriate and has been replaced by alternative regimens using recombinant molecules [26]. Even under these circumstances it is not entirely clear whether the immunobiology of this population [27], reflected for example in varying lymphocyte subsets and heterogeneity of the CD34+ cells [28] may not, with longer follow up, predicate the use of one or other peptide or, indeed, even return to the use of bone marrow aspiration [29].
Table 2

Autograft collection and infusion data

<table>
<thead>
<tr>
<th>Number and diagnosis</th>
<th>Total in bag</th>
<th>Days to peripheral blood response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mononuclear cells (&lt;10^6/kg)</td>
<td>CD34+ (&lt;10^6/kg)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>1. RL</td>
<td>7.7</td>
<td>1.08</td>
</tr>
<tr>
<td>2. SC</td>
<td>8.7</td>
<td>0.89</td>
</tr>
<tr>
<td>3. ST</td>
<td>9.6</td>
<td>2.58</td>
</tr>
<tr>
<td>4. DC</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>5. SF</td>
<td>6.0</td>
<td>22.6</td>
</tr>
<tr>
<td>6. GC</td>
<td>1.43</td>
<td>a</td>
</tr>
<tr>
<td>7. KB</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>8. MA</td>
<td>9.7</td>
<td>2.24</td>
</tr>
<tr>
<td>9. TS</td>
<td>6.3</td>
<td>1.4</td>
</tr>
<tr>
<td>10. FB</td>
<td>8.6</td>
<td>1.2</td>
</tr>
<tr>
<td>11. HR</td>
<td>8.4</td>
<td>1.2</td>
</tr>
<tr>
<td>12. CyM</td>
<td>6.0</td>
<td>1.18</td>
</tr>
<tr>
<td>13. PB</td>
<td>11.77</td>
<td>0.46</td>
</tr>
<tr>
<td>14. JS</td>
<td>12.13</td>
<td>2.08</td>
</tr>
<tr>
<td>15. DE</td>
<td>6.6</td>
<td>1.39</td>
</tr>
<tr>
<td>Mean</td>
<td>8.2</td>
<td>3.3</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Range</td>
<td>6.0–12.13</td>
<td>0.89–2.256</td>
</tr>
<tr>
<td>Median</td>
<td>7.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Patients have not to date, received their stored graft.

5.1. Donor side effects

Donor side effects were evaluated at two levels. Firstly was safety and in approximately 30% no symptoms developed. In the remainder these were mild. The incidence of lower back pain, with occasional headaches, was low and both responded to simple analgesia with paracetamol. This observation is consistent with the known effect that, even at different schedules [30], mobilisation is equivalent but discomfort decreased with dose [31]. Notably, with three years of follow-up, late complications are rare [32,33]; but, in the interest of prudence, even longer observation is required. Apart from a single instance there was no discomfort either at the ante-cubital or femoral vein site used for establishing venous access.

Secondly is the effect that this intervention on the immunohaematopoietic system itself where stimulating factors transform the marrow into a highly proteolytic environment [34]. Other relevant observations are a decrease in the more primitive population [35] and an alteration in the Th 2 to Th 1 ratio thereby providing a biological explanation for the lack of severe acute graft-versus-host disease following infusion of the peripheral blood derived harvest [36]. Less information is available on children although grafts, adequate for adults, can be obtained with donor body weights as low as 15 kg [37].

Given these caveats every effort should be made to shorten period at risk. One manoeuvre is to define response to a test dose of the mobilising molecules [38] and kinetic studies, confirmed in the present work, demonstrate that rising leucocyte and platelet count predicate optimum timing [39] with the desired progenitors being higher than when conventional bone marrow grafts are used [40]. This pattern, derived from normal subjects, distinctly changes in those who have previously
been exposed to chemotherapy en route to autologous collection since recovery is suppressed in these scenarios. A word of caution is appropriate since there is considerable variation in the harvesting schedule [41], introduction of combinations as with stem cell factor [42] and the assay techniques employed [43].

5.2. Collection characteristics

Collection characteristics depend upon intrinsic reserves, how much of this capacity remains after prior chemotherapy in the case of autografting and the extent to which release of progenitors into the circulation can be enhanced by pharmacologic stimulation. Furthermore, but not specifically examined, is the ongoing debate about potential contamination of the apheresis product by tumour [44].

Three criteria were used to define adequacy.

Firstly, based on our earlier experience (Wood and Jacobs, unpublished) and recommended guidelines, a minimum number of $6.5 \times 10^9$/kg mononuclear cells was the target [45]. Since it has been shown, and confirmed, that in normal subjects optimum numbers are circulating on day 5 collections were standardised to this time [46]. During the collection cellular content of the bag was serially monitored and, in the absence of side effects, this target was reached in a single apheresis in 33 of 48 donations with the instrument preset to process approximately five times the calculated plasma volume [47].

Secondly, additional quality control required that a minimum of $2 \times 10^9$/kg CD34+ progenitors were infused with this value achieved in each instance [48]. It has previously been reported that there is a linear relationship between the different component populations and this was confirmed in the present study (Fig. 1(a) and (b)). There is evidence that increasing number of the progenitors [49] will improve engraftment characteristics defined by the appearance of a steady increase in the rate at which granulocytes and platelets appear in the circulation up to of $2 \times 10^9$/kg but, thereafter, increments are of questionable value [50]. In this context the association of T-lymphocytes may be important. A certain number are necessary for establishment of immunohaematopoietic function [51] but there seems to be an optimum balance between intensity of host conditioning and the degree of T-cell depletion in the graft where either quantitative or qualitative differences may predominate the incidence and severity of acute and therefore, subsequent chronic graft-versus-host disease (GVHD) [52].

Thirdly, of probably greater importance, is the repopulating potential of the donation determined by colony and cluster formation of the clonogenic assay [53]. Although a number of comparable in vitro techniques exist no practical advantage attends the choice of studying the more mature cells whether these be myeloid or erthyroid but rather using an alternative assay to document long-term colony initiating progenitors [54].

5.3. Allografts

Allografts run the risk of adverse side effects and in unfractionated procedures immunosuppressive regimens typically include methotrexate, cyclosporin A and additional corticosteroids [55]. As previously reported [10] ex vivo exposure to the Campath series of monoclonal antibodies to deplete T-lymphocytes has been confirmed as an effective manoeuvre requiring no additional immunosuppressive therapy [56]. Initially the older lytic immunoglobulin or IM almost totally removed these cells but with the switch to the opsonic variant there are additional considerations including in vivo consequences which are difficult to quantitate [57].

Autografts were shown to have a different pattern in which mononuclears and CD34+ cells appeared in the circulation. Here there was a bimodal distribution with most patients behaving similarly to the allografts but a second peak becoming evident on day 7. It is currently unknown what underlies this phenomenon. It can be speculated that release is disturbed by prior damage to the inductive microenvironment [58,59] but, acknowledging that numbers were small, no distinction was evident by disease category or class of cytotoxic drug. Not surprisingly this fact necessitated second or third collections necessary to meet the minimum requirements
for graft safety. Despite this restriction the requisite number of cells were obtained in all instances.

5.4. Recipient outcome

Recipient outcome is notable that, although differences were present in donors and quite striking in the collection characteristics, the response after infusion was indistinguishable. This raises two interesting points.

Firstly, it appears as though both the quantity and the quality of the progenitors recovered, whether from volunteers or after chemotherapy and irrespective of the diseases studied in the autografts, are indistinguishable in generating short-term recovery of phagocytic function reflected in numbers of circulating monocytes and neutrophils. Furthermore, since platelet regeneration was also comparable, it can be inferred that the committed myeloid compartment is not selectively damaged although it is conceded that the more sensitive evaluation of reticulocyte response was not specifically examined.

Secondly, and as part of this ongoing investigation, is the possibility of more subtle change in lymphocyte subsets that may unmask differences in immune function as humoral and cellular pathways are studied over longer periods of time. However, preliminary analysis of patient outcome shows no difference in early bacterial or viral infection between the allografted or autografted recipients. Nevertheless vigilance is mandatory to anticipate the risk of these complications [60] and reducing them as far as possible with appropriate vaccinations [61]. In similar context is the intriguing possibility that late-presenting grade I graft-versus-host disease limited to the skin and rapidly responsive to topical or systemic steroids, may favourably impact the incidence of tumour relapse.

The hypothesis is that enhancement of a desirable graft-versus-leukaemia effect may be separable from the more commonly recognised, but deleterious, inflammatory process [62]. For example it is suggested that cyclosporin A can upregulate, in the autologous situation, immunologically mediated recognition of foreign antigen and this pathway has the potential to be selectively modified and harnessed as a means for recognising and eradicating residual neoplastic cells [63].

6. Summary and conclusion

This ongoing prospective investigation has three goals. Firstly, to evaluate donor side effects and these were shown to be minor with the procedure having high patient acceptability. Secondly, to characterise the response to the stimulatory peptide with most allogeneic donors achieving peak mononuclear and CD34+ cell populations on day 4 at which time collections took place. Differences in autografts were thought to reflect prior chemotherapy and a second increment noted on day 7 despite which quantitatively adequate recovery was obtained in each instance. Thirdly, to define recipient outcome where it was demonstrated that, for both sets of donors, peripheral blood neutrophil and platelet regeneration was similar and consistent with published guidelines. It is concluded that the programme, standardised in this way, complies with international standards of practice. In the longer-term numbers and phenotypically distinct subsets of T-lymphocytes remain to be correlated with bacterial and viral infections as well as immunologically mediated events that included acute and chronic graft-versus-host disease and the possibility of a separate cell-mediated anti-tumour benefit.

Acknowledgement

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References


The use of granulocyte-colony-stimulating factor in volunteer unrelated hematopoietic stem cell donors.

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Granulocyte-colony-stimulating factor (G-CSF) is used for the mobilization of hematopoietic stem cells in healthy donors. It has a number of common side effects such as bone pain, which resolve rapidly after administration is discontinued. Recent publications have raised concern that it might act as a trigger for the development of hematologic malignancy in susceptible individuals, possibly by causing genomic instability, but to date there is no evidence that healthy volunteer donors who receive G-CSF are at any increased risk. Ongoing studies aim to confirm whether or not G-CSF can cause chromosomal abnormalities in healthy donors. In the UK, the British Bone Marrow Registry and Anthony Nolan Trust give G-CSF to donors who have agreed to donate peripheral blood stem cells. It is recommended by the UK Registries at present that all stem cell donors are given updated information explaining the current uncertainties with regard to the use of G-CSF before they give informed consent to its administration. This information is based on a statement agreed by the World Marrow Donor Association for use by individual donor registries. Further, it is our current practice that all donors who have received G-CSF, as well as marrow donors who do not, should be under regular review for at least 10 years to allow the occurrence of any long-term adverse events to be documented.

PMID: 18373639 [PubMed - indexed for MEDLINE]
Possible harmful effects of short course granulocyte colony-stimulating factor in normal donors

The initial success of haematopoietic stem cell (HSC) transplants, intended to reconstitute a patient's bone marrow function after high dose or 'supralethal' chemotherapy or chemoradiotherapy, was based on the use of bone marrow derived from either a related or unrelated volunteer donor. However, in the late 1980s it became clear that an alternative was to collect HSCs from peripheral blood following mobilisation with haematopoietic growth factors (HGFs), either granulocyte colony-stimulating factor (G-CSF) or granulocyte/macrophage colony-stimulating factor (GM-CSF).

To a large extent, the use of mobilised peripheral blood HSC has replaced marrow-derived stem cells as the preferred source of donor HSCs. The cells collected by prior treatment with cytokines include substantially more granulocyte/macrophage colony-forming units (CFU-GM), more CD34 cells and more lymphocytes than an 'equivalent' marrow harvest.

Peripheral blood HSC grafts are particularly useful in the setting of reduced intensity conditioning allografts where transfusion of large numbers of CD34 cells may be important. Whilst G-CSF and GM-CSF are very similar or identical to cytokines produced in the human body, investigators and clinicians have been aware since their first clinical use that their administration, even in a single short course, could possibly constitute a risk for healthy donors either in the short term or as a delayed effect. For this reason the healthy donors who receive them have been subjected to extensive follow-up evaluation and transplant centres and transplant registries on both sides of the Atlantic have attempted to maintain close contact with donors for many years after their respective donations. Thus the European Group for Blood and Marrow Transplantation reported five haematological malignancies from a database of 16,431 donors who had received G-CSF [A. Gratwohl (2004), personal communication] and the National Marrow Donor Program in the USA reported four cases of malignant disease out of 2,370 donors followed for varying periods of time [D. Confer (2005), personal communication]. In both cases, the incidence of malignancy was deemed not to have differed significantly from what would have been expected in a normal population that was not exposed to HGFs [Bacher et al., 2005]. Other smaller series failed to identify any increased risk of malignancy after G-CSF administration (summarised in Pulskipher et al., 2006). In summary of the available clinical data, it seems that the notion that HGFs cause or contribute to malignancy in a normal person is very far from established.

In contrast to the short-term treatment of HSC donors, G-CSF has been used for prolonged periods in patients with severe congenital neutropenia (Kostmann syndrome) and there does seem to be some definite risk. After treatment for 6 years the projected risk of progression to myelodysplastic syndrome or acute myeloid leukaemia was 2.9%, though after treatment for 12 years the risk had risen to 8.0%. However, it is likely that such patients are constitutionally at risk of their disease progressing to overt malignancy, which could simply mean that G-CSF is a co-factor or expedites this progression. The observation may or may not have any relevance for normal persons exposed to G-CSF for a single 5-day course.

At Tel Aviv University, Nagler et al. (2004) reported finding specific abnormalities in lymphocytes from normal people who had recently received G-CSF that were very similar to those seen in lymphocytes from persons with malignant diseases. Specifically, these abnormalities included loss of synchrony in allelic replication and aneuploidy. The abnormal timing of allelic replication was a transient phenomenon but the aneuploidy persisted in the longer term. The authors stated that these changes were characteristic of changes seen in lymphocytes from patients undergoing chemotherapy for malignant disease.

The paper by Bennett et al. (2006) that appears in this issue of the British Journal of Haematology reports five cases of leukaemia, three lymphoid and two myeloid, occurring in individuals who had received HGFs. Three patients had received a pegylated recombinant human megakaryocyte growth and development factor (hHuMIGDF), which has now been withdrawn from the market, and two patients, who developed acute myeloid leukaemia, had received G-CSF in the course of donating HSCs to siblings who were undergoing treatment for acute leukaemia. However, as noted in the paper by Bennett, large-scale studies in both Europe and the USA have not found increased levels of haematological malignancy in G-CSF-treated donors.

There is no doubt that the use of HSC transplants has offered a potentially life-saving procedure to many patients. However, it must be recognised there are established and also unknown risks for the volunteer donor whatever method is used to harvest the HSC.

How then, should the bone marrow transplant community respond to this latest paper and the reported remote, but presumably finite, possibility that G-CSF could be harmful in the long-term? Most would agree that currently to abandon use of G-CSF for normal donors would not be justified. In the
meanwhile, five measures warrant consideration by organisations involved with the provision and use of donors worldwide:

1. The international transplant community and donor register organisations need to reach a consensus as to the long-term risks that donors are being subjected to and what risk is acceptable. Transplant centres and donor registries should ensure that they follow normal donors postdonation with as much precision as possible. The duration of follow-up should arguably be a minimum of 10 years and perhaps lifelong. Such follow-up should apply equally to bone marrow donors.

2. Selected normal donors should be studied longitudinally with cytogenetic analyses of lymphocytes and other tests designed to detect persisting damage both to the lymphoid and, perhaps more importantly, to the myeloid lineage.

3. Donors should be exposed to the minimum amounts of cytokines, such that the maximal number of doses and the maximal number of courses are limited.

4. Consideration should be given to long-term (more than 1 year) insurance cover of donors following leucapheresis or marrow collection.

5. Prospective donors must be told of the somewhat uncertain situation regarding G-CSF for blood stem cell mobilisation so that they can balance the risks against those associated with bone marrow donation.

The continuation of this potentially life-saving treatment will depend upon the ability of the scientific community to monitor and evaluate on an ongoing basis the level of risk associated with G-CSF administration, so that donors, both past and present, can receive and assess for themselves the best available evidence.

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References


Keywords: stem cell transplantation, granulocyte colony-stimulating factor, normal donors, leukaemia, haematopoietic growth factors.
CHAPTER 3

UNFRACTIONATED MARROW

THE WORLD STANDARD OF CARE
International experience was based on the use of marrow rich blood. This was obtained by multiple aspirations under general anaesthesia and generally with two teams working in parallel. The target was approximately 10ml/kg lean body mass of the recipient using appropriate anticoagulation. After ensuring that the recovered product was monocellular by gently passing it through a series of stainless steel screens, having decreasing pore size, samples were obtained for quality control and the graft infused intravenously. Attention was given to side-effects including changes in blood pressure and occasional oxygen desaturation.

Focus was maintained on local requirements. Nevertheless with accumulating experience each new observation or innovation was systematically tested in the laboratory utilising the established culture studies and exploration in animal models before introduction into ethics approved treatment protocols. Care was taken to integrate new information from international cooperative studies for both autografting and allografting. Examples were rejection and acute as well as the often consequential chronic graft-versus-host disease. Both generated substantial morbidity and mortality.

At this point it was appreciated that accountability was essential but such was nowhere available on the African continent. Accordingly there was the initiative of obtaining Ethics and Research Committee review and approval for scrupulously followed protocols. Clearly even this was inadequate on its own and, despite duplicating international methods, the seminal commitment was made to voluntarily report all information. This took place on every consecutive patient first to International, then autologous and latterly the Centre for International Bone Marrow Transplantation Research.

Such scrutiny with participation in these registries brings with it huge resource consumption but this is offset by three benefits. Access became constantly available to any changes that emerged with modifications by disease category. One example was severe aplastic anaemia where we had reported corresponding investigations.
It is also suggested that it is the saliva which is responsible for dental erosion and not acidic food and drinks. However, patients suffering dental erosion have been shown to have saliva of normal pH and buff ering capacity. Erosion which resulted from initial tooth decalcification was ascribed to the consumption of fruit juices, even though they were only in contact with the teeth for a short period of time before swallowing.  

The buffering effect of saliva is only modest — the pH value of the apple-saliva bolus typically ranges between 4.3 and 4.9. However, according to Jemiel, "The increased buffer capacity of saliva stimulated by chewing apples is clearly offset by their low (dietary) acid and their sugar content." The measurement of the stimulatory effect of apple products on the volume and quality of saliva has apparently not been investigated in depth, and it is not known whether the pH of the mouth reaches the critical level of 3.5. So while apples cannot be recommended as non-noticeable snack-food, they are claimed to be preferable to sticky confectionery because, gram for gram, they contain one-tenth the amount of carbohydrate, and also provide pomegranate (the fibre content), minerals and vitamins. Ascorbic acid (vitamin C), the most prominent vitamin found throughout the apple, is most concentrated under the skin.

Chewing a hard food or fruit, such as a carrot or apple, after a meal was once recommended as a way of preventing dental caries and gum (gingival) disease. This is no longer advised for carrots. As for apples, although the work of Slack and Martin in 1958 suggested that eating them slightly reduced the incidence of tooth decay, and that the gums of apple eaters were thereby improved, subsequent workers have concluded that eating apples does not enhance the health of gingival tissues. Instead, it removes plaque only from the smooth surfaces of the teeth, while inter-dental and gingival plaque is unaffected.

In a recent review it is recommended that "apples must be demoted from their position of prominence as foods good for teeth." We suggest that further investigations are needed into the properties of fresh and processed apples in order to establish whether the fruit, as a food, is in fact beneficial or detrimental to the mouth.

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Bone Marrow Transplantation

Peter Jacobs

Both experimentally and in clinical practice the replacement of an irreversibly damaged organ offers a logical and life-saving approach to treatment in a number of threatening situations. Unfortunately, biological systems provide barriers far greater than the simple mechanics of removing, for example, a diseased kidney or heart and substituting this with its healthy counterpart. The most immediate problem encountered is that of rejection where antigenic disparity between donor and recipient may lead to loss of function in the foreign tissue. The administration of immunosuppressive drugs to try and avoid or suppress this complication frequently compromises the immunologic competence of the host, thereby predisposing it to lethal opportunistic infection.

In bone marrow transplantation the situation is more complex, since the graft contains a mixed population of cells. On the one hand are the haematopoietic stem cells, precursors of blood, that migrate to the marrow cavity, grow and eventually enter the circulation. On the other hand are contaminating, immunologically competent lymphocytes. The latter recognise the antigens of the recipient as 'non-self' and respond by mounting an inflammatory reaction directed against skin, gastrointestinal tract and liver—the unique syndrome of graft-versus-host disease.

Despite these two formidable challenges the last decade has seen progress in some aspects of bone marrow transplantation and there is currently increasing use of this procedure in a number of conditions previously considered to be uniformly lethal. Already there is clear need to extend the use of bone marrow transplantation, the extent of which will be determined by the success with which the technique is used.
rejection and other complications can be controlled.

Indications and results

The bone marrow is susceptible to damage by genetic, immunologic and toxic mechanisms. Where this is partial, recovery can occur and it is necessary diligently to seek out this possibility in each individual. However, where organ failure is total the term aplasia of the marrow is used. This is a more ominous situation since randomized prospective studies have established that bone marrow transplantation is the preferred form of treatment whereas response to the administration of male sex hormones, known to stimulate blood formation, has no statistically significant benefit.

In aplastic patients being considered for bone marrow transplantation the likelihood of the damage being reversible should be excluded by a two week period of observation during which time all the necessary preparations are being made. Persistence of severely impaired blood formation beyond this time, characterized by low red cell, white cell and platelet counts, identifies a patient population having a high mortality and here it is rational to proceed to transplantation.

In the marrow, developing blood cells are normally nourished from a scaffold of non-haematopoietic tissue known as the inductive microenvironment. Theoretically, therefore, haematopoietic failure may arise from defects in either of these two components. Convincing demonstration of environmental failure is based on animal studies; proof is lacking in man. Currently, most experimental evidence incriminates the haematopoietic stem cell. Recent interest has been centered on the possibility that impaired function may be immunologically mediated rather than due to intrinsic damage. To identify these individuals in vitro marrow culture is used; function may then be returned to normal by the administration of immunosuppressive agents. Unfortunately, the majority of patients require replacement of irreversibly damaged stem cells and transplantation offers a cure rate, in a previously lethal disease, of around 50%.

A similar situation prevails with children with severe immunodeficiency diseases in that, when a donor is available, transplantation is no longer a contentious issue. As in severe, acute aplastic anaemia, a plea is made for this form of treatment to be considered immediately on diagnosis. The well intentioned practice of instituting periods of blood transfusion or white cell and platelet support, in the naive hope that spontaneous remission may occur, is to be deprecated. Persistence with this unfortunate course of action leads to the development of isoinmunization and consequent loss of either the transplant option, substantially increased risk of rejection, or the development of graft-versus-host disease.

Depending upon circumstances, cure rates of over 50% are realizable. Leukaemia is a third case where bone marrow transplantation may be used. Initially, only patients who had failed to respond to conventional drug regimens or who had relapsed were referred for this form of experimental treatment. Many of these patients had been exposed to a wide variety of cytotoxic drugs over prolonged periods of time and were in the terminal stages of their disease. The salvage rate of between 10% and 15%, although low, is distinctly encouraging when the patient population is considered.

The latter results allow of two comments when considering transplantation in the leukaemic patient. Firstly, this procedure remains a viable therapeutic option even following relapse. However, it is essential that drug therapy should not be persisted with and that patients undergo the procedure immediately a second complete remission is achieved. By following this self-evident approach, complications arising from cumulative drug damage can be avoided.

Secondly, and of perhaps greater interest, is preliminary evidence to support the use of marrow transplantation, whenever a compatible donor is available, in adults achieving their first complete remission. In adults recent data demonstrate remission rates of just over 60% at two years, a figure which compares favourably with the best obtainable with drug treatment.

The same choice should be made available to children with acute lymphoblastic leukaemia in the presence of bad prognostic factors such as mediastinal or disease of the central nervous system. In both these groups chemotherapeutic regimens are generally acknowledged to be poor and no individual should be denied this alternative option. Results with these patients are presently inadequate for analysis because too few have been transplanted.

Marrow transplantation is possible in other situations but here even less clarity exists. For example, it is uncertain whether constitutional or familial anaemias would qualify in view of the possibility that siblings may share the genetically determined defect. Furthermore, because morbidity and mortality are substantial, patients with lethal genetic diseases and those with chronic granulocytic leukaemia should be evaluated for transplantation only on protocol study by groups committing their research and service resources to this form of treatment as a major priority.

Transplantation techniques

The transfer of marrow between identical twins, syngeneic transplantation, is the ideal practice since antigenic disparity does not exist. Understandably, rejection and graft-versus-host disease are not encountered, so that results obtained with aplasia and leukaemia are superior to those where the donor was not a twin.

In allogeneic transplantation, where the donor is a brother or sister, preoccupation is with selecting the sibling who, using laboratory tests, has the identical tissue type. This compatibility is defined by a set of genes carried on chromosome 6, which code for antigens on the surface of leucocytes designated as the major histocompatibility complex. Despite apparently perfect matching, rejection and graft-versus-host disease still occur and both phenomena may therefore reflect the influence of immune reactive genes located outside the strict confines of the HL-A system.

On the basis of such minor degrees of incompatibility, high doses of cytotoxic drugs are administered, with or without additional radiotherapy, in an attempt to suppress in the recipient immunologic responses to donor antigens. To some extent these increasingly aggressive regimens are offset by a higher incidence and greater severity of graft-versus-host disease and infection.

The transplantation procedure itself is relatively simple. Under general anaesthetic the donor has marrow collected from the pelvic and sternal bones by simple aspiration with a needle. A total volume of between 500 and 700 ml of marrow-rich blood is harvested, which contains between 1.5 and 5.4 x 10^9 nucleated cells and an adequate number of haematopoietic stem cells to reconstitute marrow function in most individuals. The adequacy of the graft can be monitored in vitro by bone marrow culture, although such a test is not a guarantee of engraftment.

Autologous bone marrow transplantation requires separate comment. Here the patient's own marrow is collected, stored and later reinfused intravenously when the necessity arises. Clearly this option will not exist in the patient with aplasia but it offers an approach for those with leukaemia having no suitable donor and for a variety of non-haematologic malignancies where treatment is limited by iatrogenic marrow damage. This treatment in patients with leukaemia is fundamentally defective since the graft is likely to be contaminated by members of the original malignant clone. However, if immunologic techniques can be developed for the recognition and removal of these tumour cells, then the possibility of infusing a 'clean graft' is attractive, since it carries with it no risk of rejection or graft-versus-host disease. These theoretical concepts are the basis for persisting with the evaluation and development of autologous bone marrow transplantation: the coming decade should see this technique placed in clearer perspective.

Cell support

In addition to the transfer of bone marrow stem cells from donor to recipient, the necessity to protect patients during the
period immediately following transplantation requires the availability of effective white cell and platelet support. Such facilities, involving the separation of blood fractions, are an integral part of the modern transplantation centre. The blood components must be readily available and derived from procedures where the highest degree of quality control has been established and is constantly maintained.

There is no doubt that the infusion of adequate numbers of functionally intact platelets has reduced morbidity from haemorrhagic episodes. As this problem has diminished so its place has been taken by infection, which emphasizes the need to provide reliable granulocyte support. The development of filtration and continuous flow techniques for harvesting sufficient number of white cells from donors remains the subject of intensive investigation. These are best considered research projects linked to carefully controlled protocols for bone marrow transplantation.

**Graft-versus-host disease**

This unique immunologic phenomenon occurs only in the recipient of a successful marrow graft and is thought to be mediated by surviving, immunologically competent donor lymphocytes. It remains the major stumbling block to the wider use of allogeneic bone marrow transplantation. Two clinical entities are recognizable. The acute syndrome characteristically occurs within four weeks of transplantation, runs a fulminating course and has a very high mortality. At present it is uncertain what role the lymphocytes present in the graft play in this variant of disease; the early onset suggests that they may be implicated in pathogenesis. In contrast, a more chronic inflammatory condition of skin, gastrointestinal tract and liver occurs later after transplantation and has been ascribed to cells, probably lymphocytes and monocytes, originating from the established graft. In either event failure to recognize the aetiological mechanisms has bedevilled management. Prophylaxis remains the cornerstone of treatment and on the basis of results obtained in syngeneic and autologous transplantation has focused attention on the need for more precise matching. Once established, attempts to modify severity of the graft-versus-host disease with immunosuppressive agents and antisera directed against the lymphocyte have met with only limited success and many theoretically hasten the progression of the inflammatory process. Of interest are experimental observations that, in animal models, the administration of immunologic adjuvants such as Cyclosporin A and Corynebacterium parvum both abrogate the phenomenon and prolong the survival of transplant recipients. It now remains to test their efficacy in man.

**Summary and conclusions**

Bone marrow transplantation is established as the preferred form of treatment for patients with severe aplastic anaemia and immunodeficiency diseases. This procedure is contingent upon the availability of a willing and compatible donor. It is impossible to over-emphasize the importance of referring potential patients early, before isoinmunization has resulted from blood transfusion and thus compromised the chances of success.

In both adults and children with acute leukaemia, cautious optimism is justified by available results. In both groups a small but significant salvage rate is possible even in patients with drug-resistant disease or following relapse. Preliminary data support the use of transplantation in first remission as the primary form of treatment, in both adults and children who have disease markers for poor prognosis.

Major problems still persist. On the one hand autologous complications such as isoinmunization should be reduced as the education of physicians results in a better appreciation of what early bone marrow transplantation will offer. In contrast, graft-versus-host disease, or 'reverse rejection', is not understood and stands out as the greatest obstacle currently preventing the wider use of this form of treatment.

Clearly, much progress has been made and there is every reason to persist with research programmes seeking to bridge the gap between what is theoretically and experimentally possible and what current clinical experience has shown can be realistically achieved.

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**Crocodile Research and Conservation in Southern Africa**

**J. P. Loveridge**

In July this year the Crocodile Specialist Group (CSG) of the Survival Service Commission of the International Union for the Conservation of Nature (IUCN/SSC) will hold its fourth meeting in Miami, Florida, to review progress in the recent worldwide moves to conserve crocodilians. The first such meeting was held in New York in 1971, and now, nearly ten years later, seems an appropriate time to review progress achieved towards the stated aims of the CSG in Southern Africa. These aims include the review of existing knowledge of the status of the 21 species and subspecies of crocodilians, to determine national and international research, conservation and management priorities; and to devise a mechanism for co-operation and co-ordination of effort in these fields. In Southern Africa south of the Zambesi and Cunene rivers only one species of crocodilian, Crocodylus niloticus Laurent, now lives. The Nile crocodile is therefore the subject of this review.

**Distribution and present status**

Zoologists might be excused for expecting that the distribution of such a conspicuous species as the Nile crocodile should be well documented. In this they will be disappointed for four recent authors give distribution maps which differ substantially from another, and none of which is correct in the detail of distribution in Southern Africa. The probable former distribution of Crocodylus niloticus is given in Fig. 1, from which it can be seen that it was absent from Lesotho and the Orange Free State, and from the arid areas of Namibia, Botswana and nearly the whole of the Cape Province. Sizable populations formerly existed in Zululand, with fewer individuals in the larger rivers of the Natal south coast, Transkei and the eastern Cape. The species is now extinct in the Cape Province and, though never plentiful, was once found as far west as Uitenhage and occasionally in the Keiskamma river mouth. In the Transvaal, crocodiles were formerly numerous in the rivers of the eastern portion and in the Limpopo, Swaziland, Mozambique, Zimbabwe and Zambian rivers supported substantial populations of crocodiles and in Botswana, the Chobe and Okavango swamps were refuges which connected through the Caprivi strip with the Zambezi and Cunene populations. Since the last survey, the status of crocodile populations in all the Southern African countries must give in-
Overview of Bone Marrow Transplantation in South Africa

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Historical settings of present trends

The 30 million South Africans are as heterogeneous as the geography, climate, fauna, and flora that make up the southern part of this continent. This diversity is translated into substantial differences of cultural, political and delivery of health services. Historically the tribal or traditional healers functioned as primary care physicians and their practices currently continue to a greater or lesser extent. This influence is acceded varying recognition by those trained along western medical lines but, interestingly, there is a likelihood that statutory bodies will soon recognize and reimburse their contribution to all facets of contemporary medicine. At the other end of the spectrum are the conventional physicians working with a broad range of paramedical professionals that prominently include nurses. These multidisciplinary programs link the public and private sectors. Unfortunately, the latter are not yet participating appropriately in academic functions such as teaching and training although there are encouraging signs of an expanding role in good quality research.

It is against this background that the rapid and wide-ranging national changes taking place in this country need to be seen. There is now an overdue redistribution of available money under way to extend primary and secondary level services to peripheral areas. Inevitably there will be a reduction in commitment and size of tertiary activities which are, by nature of the sophisticated technology involved, disproportionately costly when the global needs of any community are considered. Debate rages on how best a shrinking budget can be reallocated to meet the present policies of the new government. An illustrative example of the ongoing exercise is that of bone marrow transplantation.

The stimulus to introduce these procedures into South Africa, nearly a quarter of a century ago, was 2-fold. The first was an appreciation of how experimental hematology was being transferred to the clinic. The melting-pot for these activities was a group that has become the International Society for Experimental Hematology. Amongst pioneers were E. Donnell Thomas, G. Santos, E. Cronkite, G. Mathe, and many other distinguished investigators. From this convivial environment emerged the demonstration that biological principles, such as histocompatibility testing, could be applied in a way that made possible the transfer of stem- and
progenitor cells to an individual with bone marrow failure to effect a cure. Projected techniques were cryopreservation and autologous grafting. Another visionary goal was the attempt to bridge immunologic differences between donors and recipients: this is being realized with the creation of registries for unmatched volunteers. A third area of effort was the attempt to control lethal toxicities such as graft-vs.-host disease (GVHD) and rejection.

Future requirements of hematology and BMT

Second, was the inescapable conclusion, from my years spent as a fellow at the University of Washington, that these procedures were soon going to become the focal point of hematology management. Here, noticeably, South Africa lagged behind the rest of the world.

The chance to capitalize on improving this shortcoming came, somewhat unexpectedly, with appointment to the newly created Chair of Haematology at the University of Cape Town. Although this school was known for studies in hemostasis through the prominence of Clarence Merkley and Hymie Nossal, the challenge was to anticipate and plan for future requirements, perhaps even at a national level. In so doing, it was necessary to avoid duplicating or even re-inventing the wheel. To avoid the academic and practical mistakes of the past, notably those of T. Bothwell and his group working on iron metabolism or A.R.P. Walker systematically investigating nutrition. What better way than to tackle the problem of bone marrow failure where a clinical need could be fulfilled in parallel with developing a role for marrow transplantation in seeking cure for leukemia, lymphoma, and myeloma. Additionally there was the attraction that, from such a patient-base, more fundamental aspects of hematopoiesis could be examined using experimental methods in the laboratory.

It is interesting to reflect on some of the steps taken to establish the program in Cape Town and then summarize the status of its counterpart developed in Johannesburg.

The first moves were into the unchartered field of radiobiology and took the form of allografting between 2 rabbit strains based largely on the work of B. Speck and A. Gratwold in Basel. Once the technique had been refined, the model was used to standardize methodology that had already been worked out elsewhere. Here variables included the cell dose needed for engraftment and an assessment of the repopulating potential that necessitated the establishment of clonogenic assays. Transplantation across histocompatibility barriers revealed the extent to which morbidity and mortality occurred from acute and chronic GVHD and led to early involvement in the use of cyclosporin-A.

As these procedures became established, challenges arose from other quarters. Of these the most daunting was a striking apathy amongst peers for what was widely regarded as a highly questionable procedure. Happily the day was saved by Dr. C. Barnard whose successful heart transplants and personal endorsement sustained our fledgling activities. Once access to this isolation facility was obtained, cell separator technology was introduced into the country, primarily to provide granulocytes for the neutropenic individual. The guidance of J. Porter Hester in this regard was invaluable. This has permeated other centers and also the commercial blood banks.

Initial problems in establishing transplant technology is SA

The scene was set and the anxiety as well as excitement at finally being able to join the international group of transplanteers remains a memorable recollection. Undoubtedly the same feelings occur as each new center is opened up, but, as opposed to the United Kingdom, Europe, and the United States where expertise is readily to hand, such was not the case in Cape Town on 11 December 1974 when the first allogeneic bone marrow transplant took place. Unfortunately this modest achievement attracted unwelcome media publicity that fuelled the animosity already existing for such new interventions especially as the first financial constraints were starting to appear on the horizon. Nevertheless, the learning curve had begun and very rapidly the problems common to all such procedures in the form of graft rejection, sepsis and the dreaded GVHD became strikingly evident. One tends to forget, with the passage of time, the many patients who died as a result of these complications but they were a constant reminder in those days, at morbidity and mortality rounds.

Another consequence was the difficulty in keeping nursing staff and the reputation grew that allografting was not much different from a death sentence. A second period became discernable when the addition of cyclosporin-A was aimed at blunting the severity of this unique immunologic phenomenon. Our animal studies had suggested that the fungal immunosuppressant had a role, perhaps in combination with methotrexate: this was supported by experience from a number of clinical centers. Nevertheless, and still true today, is the fact that the use of unmanipulated marrow, even from a matched sibling, has a significant incidence of GVHD with the attendant complications. Although we were the beneficiaries of visits from many experienced transplanters with encouragement always available from D. Thomas and B. Gale, a seminal event was the opportunity to become associated with H. Waldmann and G. Hale at Cambridge in studying their Campath monoclonal antibody; these efforts are still active and have already spanned close to a decade. Conceptually this protein, by binding to receptors expressed prominently on the T-lymphocytes believed to mediate GVHD, would provide an immunologic technique for purging the patient of the cytotoxic population. Initial studies with the mycotic variant confirmed these observations in the rabbit model, but clinical experience was relatively limited since it was superseded by the opsonizing form designated Campath 1G. My own effort was made largely in evaluating exposure of the graft to the antibody in vivo, or as it is popularly known, "in-the-bag" and this maneuver has virtually abrogated GVHD. Interestingly, when combined with in vivo administration there is a presently unexplained slow engraftment rate and when it is used only in the recipient, but without exposure in vitro, GVHD still occurs. These various permutations have the potential for different therapeutic applications. One very encouraging, but unex-
plained finding, is the low rejection and relapse rate with the *ex vivo* exposure, but small numbers and relatively limited follow-up require confirmation before this can be considered a clear benefit.

**Influence of changing national budgets on specialized health practices**

The Cape Town program center upon the multidisciplinary group that spans the various subspecialties. Only in the last few years has it been possible to commission a transplantation facility having laminar flow rooms with the influence of overseas colleagues again evident in that Dr. J. Sanders from the Fred Hutchinson Cancer Center in Seattle visited to share in the planning.

Until a few years ago our recipients have been limited to adults but children are now included. The most common indication is acute myeloblastic leukemia in first consolidated complete remission (CR): this is followed by idiopathic aplasia and Fanconi's anemia. Autografts are approximately equal in number and have comparable outcome to allogeneic transplant. The average rate is between 10 and 12 procedures per year, curtailed by histocompatibility matching particularly from the black population whilst financial constraints continue to limit availability of nursing staff needed to operate the protected environment to capacity. Given these limitations, matched unrelated sibling transplants with their higher complication rates, have so far not been undertaken. To this must be added the rapid change in philosophy where spending of the health budget should be targeted, which may further reduce the level of all high-tech programs. Consequently the interesting caveat, that, as is standard practice elsewhere in the world, expanding collaboration will be needed with the private sector. Thus, insured patients already have access to comparable management programs and this may release more public beds to indigents.

The second program is that commenced in 1983 and headed by Dr. W. Bezwoda in Johannesburg that within 5 years had reached an average of 10 procedures a year. This level is short of the anticipated 3-fold greater number that would be predicated on the basis of the patient population, but constraints include limited donor availability particularly amongst black nationals where matching is often only partial. As a result it may well be that 1 area for development is the expansion of the national donor program: again costs and the philosophy of mismatched or even matched unrelated allografting await clarification under the new health care scheme.

The team of physicians, nurses, and scientists operates out of a series of isolation cubicles and single beds supported by admission privileges to a large medical unit in the hospital. There has been a steady escalation in transplants which total 97 in 107 patients with the majority accounted for aplasia and acute granulocytic leukemia. Roughly half the patients are currently alive.

**Development of autografting since 1989**

A particular strength of this group has been their development of autografting where, since 1989, 346 procedures have been carried out in 309 patients mostly for Hodgkin's disease and breast cancer. Some 72%/ of 233 patients are alive and transplant-related mortality is low. Not surprisingly such an approach is attractive and here the source of stem cells has gradually shifted from marrow to the peripheral blood. A notable contribution is the cost-effective technology used for harvesting and reinfusion without cryopreservation when high-dose chemotherapy is used in patients with clinically localized breast cancer but with acknowledged poor outlook due to multiple node positivity. In contrast to conventional therapy with its high-relapse rate within 2 years, their current experience using escalated cytotoxic regimens with autologous salvage supports a benefit from the latter regimen. Notably the advance- ment of this program to young patients as first-line therapy, rather than rescue, has an increased response rate and superior survival. This appears to be the first such attempt to randomly compare the 2 treatment groups and the results continue to be encouraging.

In both Cape Town and Johannesburg the clinical services are complemented by laboratory studies. These are wide-ranging and include recognition of multiple drug-resistance, separation of malignant cell populations from normal as well as long-term marrow culture to expand suitable precursor populations. Other investigations focus on the use of cell lines with distinctive adhesion and growth promoting properties and in vitro studies of interleukins or different growth factors designed to elucidate benefits that can be transferred to the neutropenic or thrombocytopenic patient.

**Present status and future considerations of BMT in SA**

Considering the way these activities have evolved and, given the need to develop them in an essentially third-world setting, the present status of both program does credit to the directors and members of each team. The standards are endorsed by acceptance of data by the International Bone Marrow Transplantation Registry.

A logical conclusion would be that the areas represent distinct pockets of first-world medicine and efforts should be made to preserve them even as financial redistribution favoring primary health care is implemented. Thus, as never before, there is a need to broaden the working collaboration with the private sector and so bring these potentially curative therapeutic options within the reach of as many patients as possible. This will require the subjugation of personal and often self-serving perceptions to the wider need of the community. Specifically, both the Minister of Health and the Director General have emphasized their priority to see that those with appropriate insurance increasingly receive treatment in the expanding and readily accessible academic centers in private clinics. Only in this way can the limited slots available in state hospitals be properly allocated, with central and local government having the particular responsibility for the indigent community. This point needs strong reemphasis since calm judgement must prevail: for this to happen a
Autografting in Solid Tumors
Important Questions

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Introduction
Breast cancer is currently the most common indication for high-dose therapy (HDT) and autologous transplantation in the United States, both in the poor prognosis adjuvant setting and as therapy for advanced local or metastatic disease. Other solid tumors, particularly ovarian, small-cell lung, testicular and brain cancer have been treated with HDT and autografting less consistently over the last 8-10 years. In preparing this brief introduction, I have chosen to focus on the important questions which relate specifically to the use of HDT and autologous transplantation in solid tumors.

To summarize:
1) What are the important questions?
2) What preliminary results are available?
3) Is the basic concept of dose intensification correct and proven in solid tumors or in other diagnoses?
4) What is the source of relapse after autologous transplantation and high-dose therapy in solid tumors?
5) What is the incidence, level, and nature of tumor contamination in autografts?
6) Which source of stem cells should be used?
7) What is the role of tumor purging either by positive stem cell selection or by negative selection of tumor cells?
8) Is a graft vs. malignancy effect important?
9) Will a single high-dose therapy be enough?

Preliminary Results
Much of this discussion will of necessity focus on breast cancer since most reported studies are in this disease. In metastatic breast cancer early and extensive studies of various types of HDT and autologous transplantation have yielded a high incidence of complete remission which has in general been poorly sustained. Studies began 8-10 years ago. A small proportion of patients, variously estimated between 5-15%, have become long-term survivors after HDT for metastatic breast cancer. Selection on the basis of relatively limited disease or complete response after conventional induction chemotherapy may increase that sustained disease free survival rate to 30 or 40%. This probably represents patient or case selection rather than overall efficacy. Other studies in the adjuvant setting suggest greater efficacy. This study by Peters, in poor prognosis Stage II breast cancer, is historically controlled with an attempt to use equivalent cases and suggests that disease-free survival may be improved from 30-40% to50-70%by using single HDT and autologous bone marrow and blood cell transplant. Two major prospectively randomized Phase III studies in the same adjuvant setting are in progress but results will not be available until 1999. Another small Phase III study of adjuvant therapy by Gianni, reported at meetings but not yet published, suggests a similar improvement in efficacy.

Future Prospects
There has been major debate whether or not HDT is justified, particularly in breast cancer. Some studies of intensification of conventional chemotherapy suggest no improvement with increased dose, but have been criticized because they may represent dose de-escalation in the low-dose arm rather than dose intensification in the high-dose arm. The dose intensification achieved with autologous transplantation is much higher than that achieved with conventional chemotherapy. There is no indication that transplantation is any more toxic than conventional chemotherapy, although it is more time consuming and expensive.

References
Hematology on the African continent

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Introduction
The Zimbabwe ruins typify the mystery that surrounds the origin of advanced civilisations that existed in bygone centuries on the dark continent. Rock paintings of exquisite beauty and infinite detail tell stories that are recounted around camp fires and add to the mystique attributed to great traditional healers. Indeed, in 1997, the rural nature and cultural heritage of tribes across the length and breadth of Africa still center on the investigation and treatment of blood disorders by the “sangomas” with customs that are frighteningly alien to the western mind. But what of haematology in the traditional sense as it is perceived of in the so-called first world with a contrasting tradition of university training and degrees, clinical research, or laboratory-based studies? Nearly two years of intensive data-gathering have yielded precious little in the way of usable data. In many areas, despite persistence and repeated efforts, contact has been impossible. In others, information has been sparse and, with few exceptions, there emerges a depressing uniformity of gradual deterioration of those standards that had prevailed in earlier colonial times. However, a number of colleagues have gone out of their way to respond and it is these contributions that have provided the basis for this overview.

Perspective
Emancipation is a wonderful word, and independence as well as equality are highly desirable ideals. Realities are, however, different and a global picture of Africa, as anyone following world news on television or in newspapers can readily perceive, is one of escalating violence, political upheaval, and an instability that inevitably diverts attention away from interesting—indeed fascinating—clinical problems to a priority for survival of whole communities—let alone individuals. Superimposed upon this backdrop are the multiple agendas of political parties or governments. The latter frequently have shifting priorities so that infrastructure is under constant revision. One prominent example is the greater delivery of primary care and secondary services so that the high technology or tertiary centers are seen to be under threat. To compound the problem there are changes between the role of universities and technikons while everywhere there is a need for affirmative action incorporating the revamping of entire educational concepts. All this is taking place against an enormously wide spectrum of what constitutes even rudimentary health care. Thus in vast areas of the land mass, these facilities are primitive and focus on the management of casualties from massacres and genocide. As a result, expenditure of precious foreign exchange is earmarked for humanitarian considerations such as providing shelter and subsistence level feeding.

It is against this background that the existence of scattered pockets of first-world activity are observed. It is to these that we turn attention in the hope that they will be nurtured and survive to sustain undergraduate teaching as well as postgraduate programs. Additionally, they need to be developed to provide a suitable environment for technological growth and it is to be hoped that prevailing wisdom is such that resources will be assigned to protecting and ultimately leading to the regrowth of a few programs that, by international peer review, might contribute relevant knowledge to the investigation and understanding of hematopoiesis. Most of the ongoing research is at best descriptive patholog of or a clinical nature whereas research in cellular and molecular biology—the buzz-words of modern medicine in general and hematology in particular—is depressingly rare.

It is probably impossible to look at the African continent—or any other for that matter—and generalize. A more achievable alternative has been this attempt to indicate the status of training programs, the level of technology where this can be judged, and to draw attention to any research or development. Purely as a matter of convenience those areas where responses have been obtained are outlined by country.

Nigeria
Hematology training is provided at the university college of Ibadan, Lagos; Ilorin and teaching hospitals at Obafemi Awolowo, Ahmadu Bello, and Jos. Perhaps the best established of these is the university college hospital at Ibadan founded by Professors Luzatto, Essien, and Essen, who carried out their early work here on glucose 6 phosphate dehydrogenase deficiency and its interaction with malaria. Current interests center on thrombosis and hemostasis research on problems encountered in oncology and sickle cell disease. Training is by rotation through the four major pathology disciplines and a year in a field of interest before
writing the postgraduate examinations, which are in their traditional two parts. Successful candidates may register with the West African College or the Nigerian Medical College, both of which are postgraduate bodies. Sadly, like every other part of the public sector, there appears to be a gradual but progressive decline in standards and facilities. This is associated with the low morale and academic frustrations that have fueled the ongoing emigration. Few lecturers remain who try to maintain standards and these colleagues deserve all the support and encouragement that our society can provide.

**Kenya**

Professor Edward G Kasili, who recently died and will be sadly missed, wrote that there were no hematologists actively engaged in experimental work; indeed, he stated that their research capability was so restrained in scope that the country would be unable to participate in any collaborative project. At a recent psycho-oncology conference run in Nairobi, at which there were a number of overseas participants, it became clear that this was not an unduly critical viewpoint. Thus, after speaking to a number of colleagues, it emerged that post-graduate training programs in hematology were virtually nonexistent and any sort of investigation or management required referral of patients beyond their borders to either South Africa or not infrequently to the United Kingdom or the United States of America.

**Zimbabwe**

**Service**

Dr. McCleod E. Chityo, Dr. Alison M. Coutts and Professor Lorraine Levy report that there are currently three trained hematologists in the country and four physicians or pediatricians with an interest in hematology. Specialist services are not available outside Harare and Bulawayo. There are no specific hematology units in any hospital with care being provided in conjunction with the Department of Medicine at least at the Parirenyatwa Teaching Hospital in Harare.

**Undergraduate training**

**Medical**

Medical students have exposure to the subject in the second, third and fifth year in the M.B., Ch.B. course.

**Teaching**

The hematology establishment is one professor and six lecturers (with only two in-post at present) for 80 to 90 students with the intention of increasing the student intake to reach 200 annually. Chronic under-staffing constitutes a major problem.

The University of Zimbabwe runs a diploma course for medical laboratory technologists which is being upgraded to a Baccalaureate in science. Lecturers are few in number so that standards are difficult to maintain let alone upgrade.

**Postgraduate training**

There are currently no facilities or structured courses primarily for want of appropriate teachers. As a result, Zimbabweans train outside the country but it is uncertain how many are likely to return to provide clinical service. The university provides a 2-year postgraduate programme in general pathology with few hematologists emerging.

Nursing and paramedical professionals are not catered to at the present time.

**Research**

Cooperation between the university departments of hematology, medicine, and pediatrics focuses on epidemiological and clinical studies defining the incidence and pattern of hematological malignancies in the country but coordination and long-term projects are precluded by the staffing difficulties. Commendably, after initial difficulties, a cancer registry was started several years ago to better define the magnitude of the problem in this country, and is functioning well.

A heavy service and teaching load prevents the ample material from being utilized in any constructive way. Interestingly, there are adequate quantities of sophisticated equipment but is under-utilized because of staff shortages and limited funding. Despite this there is a home treatment programme for patients with hemophilia that is increasingly being run by their own association. More specialized management, such as bleeding or the presence of inhibitors, is centralized at a university clinic.

**Cancer**

Recognizing the existing constraints, a Committee for the Prevention and Control of Cancer has been formed to coordinate and strengthen existing activities and introduce new initiatives in a number of areas. Thus immunization for hepatitis B and educational programs about bilharzia and human immunodeficiency virus infections have been started although the tobacco industry remains a somewhat sensitive one.

Screening for carcinoma of the breast and cervix is projected but limited by inadequate funding. Early detection is being actively publicized but further investigation and management remain less than satisfactory.

Treatment with radiotherapy and oncology has focused primarily on achieving the best results with the machines available at the Parirenyatwa Hospital in Harare. A number of protocol studies have been carried out and one looks forward to the analysis and publication of data from these centers.

Palliative care through the Island Hospice, which is currently being expanded on a national basis, previously reached only a minute fraction of those requiring management and even the provision of morphine for pain control is a formidable challenge.

**Gauteng**

** Pretoria**

Professor Kenneth Stevens has commented that relatively little information is available other than that the training programs in pathology have been revised to include a larger clinical component and greater exposure to cellular and molecular biology.

Professor Geoffrey Falkson is the ranking oncologist in this country and spearheads an active group with two arms. The
first is in clinical pediatrics where the International Society of Paediatric Oncology protocols are followed and no particular free-standing projects are ongoing. In the adults there has been long association with the Eastern Co-operative Oncology Group and clinical data is incorporated in those trials but, again, no specific clinical or laboratory studies, particularly in hematopoiesis, are reported.

Johannesburg

Professor Barry Mendelow is active in a number of areas which include puromycin and cycloheximide-induced c-myc mRNA superinduction in which expression of this proto-oncogene is being manipulated to modulate regulation of the apoptotic cascade. Inherited hemolytic disorders continue to be studied, particularly at the level of abnormalities in the red cell membrane that are exemplified by hereditary elliptocytosis and spherocytosis. The work is collaborative with Dr. G Daniels of the Medical Research Council Blood Group in London and some of the projects include the identification of molecular defects, causing hereditary spherocytosis, a study of South East Asian ovalocytosis, the molecular biology of elliptocytosis in a South African kindred, spectrin and anchor binding defects as well as the molecular mechanisms involved in the invasion and growth of the malaria parasite Plasmodium falciparum in red cells. Molecular genetics focus on the mechanism of cancer initiation and progression in trisomy 12, t(3;14) in a B cell lymphoma and characterization of the 11p abnormalities in ovarian cancer. Platelets have long attracted attention and have been an area of special interest focusing currently on function, fatty acid, and cholesterol composition in type I and type II diabetes in terms of altered platelet reactivity in this context, thereby raising questions about higher platelet cholesterol levels, decreased membrane fluidity, and accelerated phosphoinositol metabolism.

With the retirement of Professor Thomas H. Bothwell, his highly respected iron metabolism unit has been disbanded. The very long years of relevant scientific contribution and innumerable publications, to say nothing of the many young scientists who received their fundamental training in his program, will be sorely missed. Clinical hematology, under the leadership of Professor Werner Bezwoda, has three major components. The first is the bone marrow transplantation service. Second is the employment of high-dose chemotherapy and autologous bone marrow or peripheral blood stem cell rescue—an area about which this group has published important controlled data demonstrating that, in the particular context of breast cancer in young women with poor prognosis, this cost effective approach increases remission rates and statistically improves relapse-free and overall survival. Third, at a basic research level, long-term marrow culture systems and adhesion to extracellular matrices, an immunobead method has been used to separate precursor cells as an approach to purging grafts of contaminating neoplasms.

KwaZulu Natal

Dr. Vinod Jogessar has reported that research is localized in two major areas. The first is the Natal Blood Transfusion Service studying gene polymorphisms in hemophilia and the use of human leukocyte antigen typing and cytogenetic analyses for inherited disorders, particularly Down's Syndrome.

Second, in the Department of Haematology, the major activities are the use of molecular techniques for documenting lymphocyte clonality in malignant lymphomas, minimal residual disease in chronic myeloid leukemia where patients are treated with interferon and in vitro interaction between hematopoietic growth factors and normal bone marrow cells. Flow cytometry is being applied to assess immune status in patients who have been infected with the human immunodeficiency virus, the phenotyping of lymphoproliferative disorders, and a study of DNA ploidy in malignant disease.

Two additional studies are the role of cytokines in the pathology of pulmonary tuberculosis and the problem of drug resistance to malaria in that part of South Africa.

Orange Free State

Professor Philip Badenhorst comments that the characterization of Fanconi's anemia is being studied since this is an area of high indemnity while there is a clinical survey in progress defining the incidence of acute leukemia and another characterizing immune thrombocytopenia in children to distinguish features that separate the acute from the chronic forms.

The Cape Province

Dr. Arthur Bird reports that the Western Province Transfusion service is studying the role of the abbreviated cross-matching in blood banking, the clinical and pathological consequences of finding hepatitis C virus antibodies in blood donors, the detection of T-lymphotropic in neonatal necrotizing enterocolitis as a basis for its management, and a tissue culture-based test for in vitro detection of pyrogens.

The University of Stellenbosch and Tygerberg Hospital were reviewed by Dr. Erna Mansvelt and Professor Peter Hesselink. There is a large routine service laboratory that includes the evaluation of patients with congenital and acquired hemostatic disorders while clinical and laboratory based research focuses on epidemiological and intervention studies in patients with nutritional disorders and contrasts population groups as a basis for iron fortification and the use of additives such as vitamin A in the pediatric population. A number of hematology projects include immunophenotypic comparison of acute lymphoblastic leukemia in children and adults, the demography and cytogenetic findings in patients with chronic myeloid leukemia, the systematic investigation of hematologic disorders in those with human immunodeficiency disease, with additional viral screening in leukemia and lymphoma. Hypercoagulability is being documented in patients with thrombosis and a short partial thromboplastin time while computer based anticoagulant control is being compared with the older manual method. Bone marrow trephine biopsies continue to be examined using microwave oven processing and the utility of sampling both iliac crests in patients with myeloma as opposed to the more traditional single biopsies is in progress.

The pediatric group headed by Professor Peter Hesselink has a well established registry (with data available for analysis from 1983 onwards) in which there are 50 or 60 new entries each year in the under 15-year-old age group. On this basis there have been a number of reports on the epidemiology of childhood cancer and perhaps the world's largest experience in the recognition, diagnosis, and treatment of onyai.
Professor Cyril Karabus reports from the Red Cross War Memorial Children's Hospital that a very heavy service load precludes major clinical studies but particular interests are in the management of hemophilia and a wide range of malignancies, including the leukemias.

The University of Cape Town established a department based in the Groote Schuur Hospital under the foundation headship of Professor Peter Jacobs in 1970. In order to support research activities, the Leukaemia Centre was created two years later. Since then it has, by common consent, played a leadership role in seeking the development of a unified discipline that incorporates clinical service, undergraduate teaching, postgraduate training and laboratory-based pathology and to further unite these through research where the theme has been the development of treatment programs that center on a multidisciplinary team giving equal weight to medical, nursing, and related professions. Attempts to propagate this concept at a national level, with standardized training and examinations, have not been successful and the current status quo of having hematopathology and clinical hematology as separate registrable disciplines has been perpetuated. Only the future will tell whether this decision, which differs from many developed countries, is the wisest way to train specialists in the changing third world that is South Africa. The feature of patient care has been the systematic study, through a series of carefully structured and peer-reviewed protocols, of hematologic malignancy with most of these culminating in bone marrow transplantation. In the latter sphere has been the cardinal observation, researched and reported in association with Professor Herman Waldmann and Dr. Geoff Hale, originally from Cambridge and now at Oxford, that T cell depletion of the graft by exposure to the monoclonal antibody Campath 1G ex vivo or in-the-bag, leads to rapid and universal engraftment, total abrogation of acute as well as chronic graft-vs.-host disease, and a promising low relapse rate in acute myeloblastic leukemia.

Cell separator technology was introduced into the country two decades ago in the same university department primarily to provide the necessary support for evolving chemotherapy programs; this technology has been expanded in a number of ways, including its adoption by the various blood transfusion services. A new observation is that serial procedures in familial hypercholesterolemia lead to the development of iron deficiency and additionally the use of inductively coupled plasma atomic emission spectrometry has shown that this reflects separator-linked extracorporeal hemolysis with iron loss in the exchange fluid and when chelated to citrate in the urine. This phenomenon was pursued using electrophoresis and it is speculated that a lipid-related acquired membrane defect, caused by high cholesterol levels, diminishes erythrocyte deformability and increases fragmentation so that shortened survival occurs as they traverse the microcirculation in vivo.

Laboratory based investigations range from demonstrating that the type of tissue plasminogen activator produced by leukemic blasts is a prognostic indicator in acute leukemia (collaboratively studied with Professor Lyn Wilson), to the introduction and development of in vitro bone marrow cultures, which have been applied to showing that exposure to chemoradiotherapy following transplantation is associated with residual damage to stroma and hematopoietic precursors. In parallel, a laboratory has been set up and equipped for cellular and molecular biologic techniques such as flow cytometry, cytogenetics, and DNA studies under the direction of Dr. Riva Rubinstein.

In the last two decades or more, postgraduate programs have been introduced and refined. These have provided training for residents in hematopathology and resulted in a steady stream of postdoctoral fellows graduating from Masters and PhD programs. An interesting and notable innovation has been, since retirement and relocation, the development of a similar university style department in a private academic complex with ongoing activity in a number of the programs previously initiated, including bone marrow transplantation using peripheral blood stem cells and laboratory-based research on the effects of T cell depletion with Campath antibodies, reporting an audit of results to the International, Autologous and European Bone Marrow Transplantation Registries, as well as the Campath Users' Group. Concurrently, flow cytometry and cytogenetics, now with fluorescence in situ hybridization, are being applied to the systematic study of hematologic malignancies.

**Hematology in a changing Africa**

These are sobering reflections that lead to two conclusions. First, where there remain vestiges of good science, such activities need to be thoughtfully nurtured. This will not be easy because available funds are increasingly redistributed to the delivery of primary health care; however, it could be reasonably hoped that careful planning will ensure a continuing role for academic programs in the context of universities and associated teaching hospitals as the indispensable guardian for standards and training in the future. Already there is an epidemic of managed health care sweeping through—at least—South Africa and it is unclear what the final composition and structure of this system, as well as research and development with its high cost and need for expensive funding, is going to be. One approach would be to favor much closer working relationships between the public and private sector where many active faculty are already relocating. In this swiftly changing environment, it is mandatory that there be cohesion in sustaining appropriate clinical and laboratory-based research programs and the time may well have arrived for private colleges and universities to be given serious consideration.

The second is in many ways an extension of the first and what is seen in Zimbabwe is a realistic reflection of the Third World, which is often quite beyond the comprehension of those trained or working in the United States and Europe. What exists is a valiant attempt by a few highly dedicated professionals to provide care with limited resources for the patients who reach central hospitals. The future is bleak since training facilities are limited, there is no incentive for well qualified, experienced staff to remain in university or government service and those who have the opportunity to study elsewhere seldom return. All this is compounded by the lack of funds needed to sustain continuity within academic departments. It is here that many of us can provide
constructive help. Some help could be at very low levels, such as donating last year’s journals to a department starved for current information, while others could offer to share in collaborative work. Also, we can use the existing patient material in association with the more advanced centers that remain to carry out cellular and molecular biology that is often needed to study unusual diseases. For larger institutions and established clinics an opportunity exists to invite colleagues to spend time participating in existing programs and to extend this through all professional ranks, whether they be doctors, nurses or technologists. Indeed the reverse is equally worthwhile and who knows what will emerge from time given up to teach a mini-course in another part of the world? Perhaps visitors will learn a little about the plight of others—a first small step that has the potential for constructive international collaboration.

Acknowledgments
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Organ replacement, including the tissue from which immunohaematopoiesis is derived, continues to expand and many lives are saved. Unfortunately, not every procedure is successful with failures attributable to rejection and, in the case of bone marrow allografting, by varying grades of acute or chronic graft-versus-host disease. Furthermore, when used to support haematopoiesis following high doses of chemotherapy, relapse of the tumour being treated can occur. These problems persist to challenge the experimental haematologist and immunologist as much as the clinician. As with most advances in medicine improved understanding of physiological principles tantalizingly promise better outcome with much of the research focused on that enigmatic primitive progenitor – the true stem cell. Unraveling the genetic mechanisms that determine positive or negative regulation may, for example, permit ex vivo expansion of this target population and so make more realistic the use of other sources that include foetal liver and, perhaps more attractively, cord blood in adults. In the interest of safety one central issue has become the definition for what constitutes a standard graft with criteria being primarily haematopoietic reconstitution. Rapidly gaining momentum is a quest to understand, and then modulate, the closely associated immunologic recovery. Here challenges include morbidity and mortality that result from a wide range of infections and the potential to harness the formidable power of the immune system for its anti-tumour effect. In an extension of the latter phenomenon it is not surprising that much effort is being devoted to non-myeloablative conditioning regimens with their perceived lesser toxicity and benefits that may be linked to chimerism between donor and recipient or further manipulations that include a donor lymphocyte infusion.

For one brief instant, when sperm fertilizes ovum, all the genetic information for subsequent emergence of that particular individual is present in a single totipotential cell. Immediately, in ways still obscure, differentiation is initiated and pluripotential systems evolve to generate each different organ within the embryo. As technology has improved elegant studies continue to define the migratory nature of the anatomical sites in which blood formation takes place [1]. Such a march of events is exquisitely orchestrated and continues until function eventually resides primarily in the red marrow. Within this tissue subtle interaction takes place between receptors with their redundancy [2] and a wide range of pleiotropic cytokines, some of which are stimulatory and others inhibitory, to create the haematopoietic inductive microenvironment [3]. Achieving optimum outcome after transplantation necessarily rests upon an appreciation of the physiological interchanges within this latter compartment.

Morphology of the candidate stem cell places it within the family of small lymphocytes [4]. However, function remains the accepted way of defining this entity. Thus, the cardinal requirement is division into two daughters with one retaining the capacity to replenish the parent pool and a second committed to progress through increasingly more mature progenitors to morphologically recognisable precursors and eventually to mature progeny. Much impetus was given to understanding these steps by Till and McCullough using their in vitro mouse spleen assay [5]. The obvious validity of the
murine model is difficult to test in higher primates and humans but extrapolation is assumed.

Clonogenic assays were introduced to explore in the laboratory those phenomena that were inaccessible to molecular dissection in the intact animal. Two broad categories exist. In one area, stimulation of cytokine production by layering the mononuclear inoculum on a preformed and irradiated stroma. This is thought most amenable to recognising the earlier or long-term colony initiating cells [6]. For a long time it was assumed that this particular sub-category would be undetectable in some of the systems. Nevertheless, they appeared to grow when proper conditions are provided that include the absence of inhibitory molecules such as transforming growth factor β [7] and contact with plastic or connective tissue substrate [8].

Flow cytometry, focusing on expression of the CD34 antigen, has added a new dimension for separation of multipotential from pluripotential compartments [9]. Recent work examines self-renewal potential with evidence suggesting that segregation is possible into phenotypically distinct sub-types both of which have multipotential capacity but differ in that some can be successfully grafted and others, under normal conditions, lack this capacity [1]. Transplant studies, linked to in vitro colony-forming unit cultures, have demonstrated a discrepancy of approximately 300-fold more activity in the marrow than detected during cell transfer [1]. Extensions of this argument focus on varying expression, even lack of demonstrable membrane CD34, and cross-talk between haematopoietic with stromal cells within the micro-environment via molecules with cytoadhesive properties [10-13].

Further ways of looking at cellular haematopoiesis include determination of telomeric length [14] and already such information may have practical application through evolving abilities to manipulate the enzyme telomerase [15]. In addition, rethinking of haematopoietic hierarchy may be appropriate so as to incorporate recent appreciation that there exists a common progenitor able to generate macrophages and dendritic cells as well as segregation of erythroid and megakaryocytic lineages from other myeloid lines [16].

Sources from which grafts can be obtained vary and, perhaps not surprisingly, reflect the nomadic pathway from early sites in the yolk sac through reticuloendothelial organs that include the liver, spleen and to finally rest in the red marrow. These sequential steps are gradually being better understood and confirm the migratory nature of both stem and progenitor cells. Such behaviour, when linked to radiobiological studies that used the shielded femur in different adult animals, drew attention to the blood as a potentially practical donor organ. Thus, while mid-trimester foetal liver appears to be a rich source of cells for clinical transplantation [17], the use of cord or placental blood may well turn out to be a practical alternative if expansion of the appropriate components can be achieved [18]. Given these circumstances it is not surprising that the marrow is becoming less favoured and being replaced by a functioning graft derived from the circulation using apheresis technology [19].

Definition of graft adequacy is the cardinal issue that governs both practicality and safety. Much of the initial experience from different centres has been refined at meetings and gathered together in the Milan–Mulhouse guidelines [20] and more recently in the International Society of Haematotherapy and Graft Engineering or ISHAGE protocol [21]. Mobilisation methods need to take into account the effects of chemotherapy with, or without, additional haematopoietic growth factors [22]. Many of these aspects are brought into perspective by the incisive analysis provided in the accompanying review by Hester [23].

Ex vivo expansion then becomes an important consideration to increase at least progenitor cell numbers [24]. It is currently less clear whether these methods are able to induce and additionally maintain the pool that is needed to engraft and sustain long-term haematopoiesis. Thus, unless this crucial non-cycling component can itself be influenced, as opposed to lineage-committed progeny, cord blood, for example, may be insufficient to durably engraft large individuals.

Haematopoietic reconstitution has been the primary target in autografting as a manoeuvre pri-
mainly to permit dosage escalation with cytotoxic drugs [25]. Here particular problems relate to deterioration in the quality of the product recovered often after many cycles of chemotherapy. This approach has nevertheless proven realistic in protocol studies for treating lymphoma [26], myeloma, acute myeloid leukaemia [27] and, though controversial in some of its aspects, efficient in solid tumours including breast cancer [28]. Further confounding the issues is contamination by small numbers of neoplastic cells and a variety of methods for purging by positive or negative selection continue with increasingly sensitive technology applied to reveal cancer cells remaining in the harvest [29]. Another application is the use of these procedures for gene therapy [30] with preliminary results in sickle cell disease [31] and Fanconi anaemia [32] illustrating some of the persisting difficulties.

In allografting there are a rather different set of circumstances to be confronted irrespective of whether bone marrow or peripheral blood is the source of stem cells [33]. These include graft rejection that may be a function of variables that range from cell numbers infused through degree of histoincompatibility to the immunosuppressive effects of the conditioning regimen where we havefavoured the use of additional body irradiation [34]. However, concern has been expressed about damage to normal tissues from this modality and a model reported that identifies some of the factors subsequently capable of contributing to the development of graft-versus-host disease [35]. The latter syndromes may be acute or chronic and significantly impair quality of life particularly evident in children [36], with consequential decrease in survival [37]. Attempts to blunt the severity of cell-mediated attack on target organs that prominently include the skin, biliary endothelium and enterocytes of the gastrointestinal tract include cyclosporin A and methotrexate. In an entirely different approach a variety of monoclonal antibodies have been used in vivo. Contrastingly, encouraging progress was reported with T-cell depletion using the Campath series of immunoglobulins ex vivo or in-the-bag and uniform engraftment coupled with complete abrogation of GVHD achieved using bone marrow [34]. Subsequent studies with peripheral blood stem cells harvested by apheresis technique, after exposure to stimulatory peptides, showed a shortened period to engraftment that was directly related to the number of CD34 cells infused [38]. Of note is that the larger number of T-cells given did not increase this complication but, for the first time in our experience, led to a low-level reappearance of cytomegaloviral seroconversion [39]. Another intriguing feature has been the reappearance of erythrodema regarded as late presenting, rather than classical chronic, grade I graft-versus-host disease and limited to the skin with our switch to the humanised version or Campath 1 H [40]. These findings, are equally applicable to matched unrelated volunteer and mismatched sibling donors [40]. This topic is penetrating the reviewed in the accompanying paper by Bunjes [41].

Immunologic recovery is fast becoming the major challenge where, historically, unmanipulated marrow not only has a high rejection rate but is associated with substantial incidence of acute and chronic graft-versus-host disease. In those clinical settings, infections with bacteria, protozoa and fungi remain a tangible cause of morbidity and mortality [42]. Thus, cytomegaloviral seroconversion has resurfaced with a switch from marrow to peripheral blood stem cells and particular vigilance, regular screening and pre-emptive treatment with ganciclovir or, as appropriate, additional foscarnet are needed to prevent progression to pneumonitis that has a high mortality [43]. Another interesting observation is that the move from the opsonic Campath 1 G antibody to the humanised 1 H version for ex vivo T-cell depletion has reintroduced grade I graft-versus-host disease restricted to the skin [40]. Whether there will be an associated beneficial anti-tumour effect or perhaps even a different spectrum of natural killer cell expression awaits clarification [44]. It is against this background that a major new area of interest is attracting attention in the form of non-myeloablative stem cell transplantation [45]. This method, by creating mixed chimerism, opens new vistas for harnessing the enormous power of the immune system without necessarily exposing patients to the hazards of high-dose chemotherapy [46]. In what might be regarded as an extension of these
innovative approaches is the subsequent use of donor lymphocyte infusion and this is documented to achieve complete remission in relapsed haematologic malignancies exemplified by chronic granulocytic leukaemia [47]. Updated information in this field is provided by Schleuning in his contribution to this topic [48].

The conclusion is that haematopoietic stem and progenitor cell transfusion is established as a clinically viable procedure in a number of different disease categories. Progress has been evident in some of the major areas that include guidelines for defining a standardised graft. Under these circumstances the pattern of haematopoietic reconstitution is predictable with differences between bone marrow and the faster recovery using peripheral blood demonstrably shorter in the latter instance by approximately one week. In autografting the challenge remains that of quality of the product collected from patients exposed to chemotherapy with its cumulative damage to the target population. In addition, the role of contamination by malignant cells is not yet solved. However, the technology has proven of value in supporting high-dose chemoradiotherapy particularly in lymphomas. Another facet is the potential enhancement of an anti-tumour effect that might theoretically be achievable by interventions that combine interferon gamma and cyclosporin [49]. Considerable promise exists for applying this approach to gene therapy but much work remains to be done in this area before it is routinely transferable from the laboratory to the clinic. In contrast allogeneic material collected from siblings, matched unrelated volunteers or partially mismatched family members focuses on the need to overcome rejection and the persisting hazards of acute and chronic graft-versus-host disease. Here the pattern of immunologic recovery is a huge challenge but still in its infancy and even the criteria that defined return to normality remains controversial. Purely the number of T-cells and their sub-sets may be inadequate and a whole new field is developing to define the repertoire of the incoming lymphocytes. Recognising that cell-mediated competence is of central importance in diminishing infectious complications and mediating tumour eradication much effort is focused on whether it is possible to segregate the undesirable consequences of host damage from the benefits of eradicating malignant cells. One encouraging way of combining the best of both goals may yet prove to be a change in technique to non-myeloablative conditioning regimens with the latter further modulated, where necessary, by donor lymphocyte infusion.

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Case report

Prolonged remission of severe refractory rheumatoid arthritis following allogeneic bone marrow transplantation for drug-induced aplastic anaemia

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Summary: Aplastic anaemia developed in a 33-year-old woman whose rheumatoid arthritis was refractory to the administration of many drugs, including penicillamine and gold. Allogeneic bone marrow transplantation reversed the haematological abnormality and simultaneously resulted in a 2-year period of relief from joint pain. Symptoms then reappeared and the serological tests for rheumatoid arthritis again became positive. The arthralgia has responded slowly to the administration of anti-inflammatory drugs and steroids. The protracted asymptomatic period may have been due to the intense immunosuppression required for marrow grafting and the subsequent administration of cyclosporin. Since she developed chronic graft-versus-host disease, the arthritis may be an unusual complication of this syndrome.

Rheumatoid arthritis may be a crippling disease in which severe symptoms require treatment with potentially myelosuppressive drugs, such as penicillamine and gold. Furthermore, prior gold therapy is described as a risk factor for subsequent development of serious penicillamine-induced adverse effects. Among the latter is marrow aplasia resulting from irreversible damage to the haematopoietic stem cells, particularly when histocompatibility antigens DR2 and 3 are expressed. The natural history of such drug-related myelotoxicity is unpredictable, but persisting aplasia may require allogeneic bone marrow transplantation, where such an option exists. Following this procedure, profound degrees of immunosuppression result from the conditioning regimens that are an integral part of allografting and this state may be compounded by the administration of additional agents for prevention of graft-versus-host disease (GVHD), including cyclosporin.

One therapeutic option in treating patients with rheumatoid arthritis, particularly when there are progressive and incapacitating symptoms, is the administration of immunosuppressive drugs. Since there is comparable but more intense impairment of both humoral and cellular mechanisms following allogeneic bone marrow transplantation, it was of interest to study an individual in whom this procedure was followed by prolonged and complete symptomatic relief of arthritis.

Case report

A 33-year-old female developed pain, swelling and morning stiffness in her knees

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and proximal interphalangeal joints 6 months before presentation. A diagnosis of rheumatoid arthritis was established, using conventional criteria, including a titre of 1/512 for rheumatoid factor associated with radiological evidence for early erosive changes in her hands. Despite administration of non-steroidal anti-inflammatory drugs, prednisone and gold salts, the disease progressed gradually and joint function steadily decreased. Penicillamine was commenced in conventional doses and after 23 months the development of neutropenia necessitated discontinuation of the drug. One month later she presented with severe follicular tonsillitis and numerous petechiae; haemoglobin was 8.9 g/dl, white count was $1.5 \times 10^9$/l, of which 77% were lymphocytes, and platelets were $12 \times 10^9$/l. The bone marrow was aplastic. Because spontaneous recovery had not occurred after 3 months of adequate supportive therapy, she was conditioned with 200 mg/kg of cyclophosphamide and underwent allogeneic bone marrow transplantation from an HLA-identical brother. Prophylaxis for GVHD consisted of cyclosporin, adjusted to maintain whole blood levels by radiimmunoassay between 200 and 400 ng/ml.36 The marrow engrafted at day 14 and only donor cells were shown to be present by cytogenetic studies and red cell phenotyping. Within one month full blood count was normal, all symptoms of rheumatoid arthritis had disappeared, and serology had become negative.

At regular follow-up there was steady objective improvement in mobility and function of all her affected joints. At 18 months after transplantation she was able to cope with housework and shopping for the first time in more than 5 years. Cyclosporin, which had been without any side effects, was withdrawn one year after grafting. The immunological status as evaluated by multi-dose skin testing, and the in vitro response of lymphocytes to mitogens and alloantigens in the mixed lymphocyte reaction, were normal. Shortly after this, the patient developed limited cutaneous GVHD (Grade I).17 Some curtailment in joint movement recurred in the course of the following year and simultaneously rheumatoid and antinuclear factors again became strongly positive. These symptoms required control with titrated doses of non-steroidal anti-inflammatory drugs, prednisone and azathioprine. At 3 years of follow-up, the patient is fully mobile and has a Karnofsky rating of 90%; synovial swelling has not recurred and, on objective testing, joint movement is only slightly limited. Furthermore, repeated cytogenetic analysis confirms that autologous marrow reconstitution has not occurred.

Discussion

The pathogenesis of the marrow aplasia in this patient appears to be causally related to drugs taken for control of relentlessly progressive and incapacitating rheumatoid arthritis. Gold salts may predispose to the subsequent development of myelotoxicity with other drugs, notably penicillamine,17 which agent is the probable cause of the development of the aplasia.18-3 at the time, she was not on any other drugs known to be associated with marrow damage or to adversely interact with penicillamine. The explanation for marrow sensitivity to drug damage cannot be attributed to the HLA antigens DR2 and 3, which are statistically related to penicillamine toxicity,9 since the phenotype of the patient and the donor was A2, A28, B44(12), B15, CW7, DR1 and DR7.

The complete remission of her rheumatoid arthritis which followed allografting is attributed to the profound immunosuppression after conditioning with high-dose cyclophosphamide. This agent is effective in treating rheumatoid arthritis,18,19 although its effect would not have been expected to persist for such a long period. There may, however, have been a potentiating benefit from the cyclosporin administered during the first year following transplantation.

Of particular interest is the clinical and serological relapse of the rheumatoid arthritis which occurred 2 years after the marrow transplantation. It seems unlikely that this is a consequence of the late re-emergence of autoreactive host lymphocytes since there is
no current evidence for autologous reconstitution and only donor cells are demonstrable by appropriate cytogenetic studies. Alternatively, although less likely, it is possible that the joint symptoms represent a component of the chronic GVHD\(^2\) that emerged for the first time when the cyclosporin was withdrawn.

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Allogeneic Bone Marrow Transplantation With a Fixed Low Number of T-Cells in the Marrow Graft

To the Editor:

Verdonck et al. have extended their earlier results on the above topic. Their approach is reminiscent of other experiences, in which T-lymphocytes are added back to a graft in which this population has first been removed using monoclonal antibodies. The reported feasibility and consequences of this manipulation have recently been commented on in some detail as a result of cumulative studies from the Campath Users' Group. Although theoretically attractive, this method undoubtedly has limitations and it therefore may be of interest that a slightly different technique, in which we expose the graft to Campath 1G in vivo, abolishes acute and chronic graft-versus-host disease (GVHD). Updated figures are given below. Thus, in 42 consecutive patients, engraftment was uniform and swift, in 30 with hematologic malignancy, rejection has not occurred and relapse rates are low. In 10 with aplasia and 2 with Fanconi's anemia, the pattern was similar, although grafts were lost in 3 requiring retransplantation (twice in 1 individual), but all are alive and completely well. Conditioning in this protocol includes 6 Gy total nodal irradiation, aimed at increasing immunosuppression. In the case of malignant disease, this modality may add an antitumor effect that, theoretically at least, might balance any graft-versus-host-leukemia (GVL) effect that could, otherwise, have been associated with GVHD.

Interestingly, the Dutch group still encountered the latter complication in 70% of their patients, although it was only grade I or II in 49%. In contrast, our experience is that even this is often unacceptable because it generates significant morbidity and, although generally underreported, a chronic performance status. Furthermore, the chronic variant is noted to occur in nearly one-third of those at risk, an additional, and far from significant, price that these individuals have to pay. Of note is a procedure-related mortality of 11%, which seems high when considering that none of the patients, in our present series, died as a result of the conditioning or transplant itself. It is possible that, in the high-risk category, these figures may be substantially worse.

Although quality of life is reported using a Karnofsky score at 1 year, this seems to refer only to standard-risk patients; again, presumably the remainder would have fared less well. Performance status is also notoriously underreported. Thus, even using well-established criteria, little real appreciation emerges for the social embarrassment created by the widespread skin lesions and the misery experienced by patients with what is euphemistically referred to as mild or limited. These issues are labored to emphasize, based on our reported data, that any degree of GVHD is necessary to achieve a GVL effect. This is certainly an area worthy of more investigation.

Thus, although the reported results are of scientific interest, we believe that the clinical experience, particularly when the substantial in-house mortality is added to the acute and chronic GVHD, needs to be seen in the light of alternatives, one of which is the much simpler procedure that we have used in Cape Town that seems able to circumvent complex immunologic manipulations of the graft and, especially if the results can be confirmed, would offer a much more practical approach to allogeneic bone marrow transplantation.

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REFERENCES


RESPONSE

The goals of our approach of partial T-cell depletion of the marrow graft (through a T-cell addback to the maximal T-cell-depleted graft, performed with a physical technique) were: (1) To prevent severe acute graft-versus-host disease (a-GVHD) but certainly not to abolish a-GVHD completely, rather to induce a mild a-GVHD and by that the graft-versus-leukemia (GVL) effect, and (2) to prevent the high incidence of graft failure occurring after maximal T-cell-depleted marrow grafting.1 Graft failure and loss of the GVL effect are the major drawbacks of maximal T-cell-depleted marrow grafting, occurring more often after T-cell depletion by monoclonal antibodies than by physical techniques.2 In our opinion, the high relapse rate occurring after maximal T-cell-depleted marrow grafting justifies the inconvenience to the patient of a mild GVHD, hopefully with maintenance of GVL. Indeed, our approach seems to have a relapse rate that is not different from that of non-T-cell-depleted marrow grafting, whereas severe GVHD has never been observed.

We do agree that the occurrence of grades I and II a-GVHD (only of the skin) in 70% of the cases and of chronic GVHD in 30% of the cases observed with our approach generates morbidity. However, the GVHD is always very responsive to corticosteroid therapy (if treatment is indicated), which is in contrast to the data of non-T-
A monoclonal antibody VCD-1, directed against the N-terminal intracellular part of the invariant chain (li) was used to show, by immunoprecipitation and Western blotting, the unprocessed and processed forms of li in chronic lymphocytic leukemia (CLL) cells, in Epstein-Barr virus–transformed normal lymphocytes (EBVL), and in cells of the Raji Burkitt’s lymphoma cell line. Terminal glycosylation and sulphation of li in the Golgi apparatus was shown in Raji cells and not in EBVL. CLL lymphocytes contain a higher concentration of p35 li than do EBVL or Raji cells.

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THE HLA CLASS II invariant chain (li) was first shown as a coprecipitant in immunoprecipitates of class II major histocompatibility complex molecules, and its expression, processing, and degradation by proteolysis have since been extensively studied. It is now known that li chains become associated with class II α- and β-chains in the endoplasmic reticulum (ER) and that this close association is maintained during intracellular transport until, in a late endocytic compartment, the li chain is digested, leaving the αβ complex free to bind exogenous, antigenic peptides for display on the cell surface.

The role of the li chain in these events is believed to be threefold. It blocks the αβ antigen-binding site, thus preventing display of endogenous peptides on class II molecules; it maintains the αβ chain complex in an immunologically effective conformation; and it targets the αβ chains to an endocytic compartment.

Thus, the invariant chain is a potentially important participant in B-cell function, particularly as this involves antigen presentation to CD4+ lymphocytes.

Electrophoretic studies have identified several forms of li that differ in charge and mass. These represent the products either of translation from two different AUG initiation codons (giving rise to 33-Kd and 35-Kd proteins) or of alternate splicing of the mRNA (giving 41-Kd and 43-Kd forms). During subcellular passage, glycosylation, and sialylation, limited proteolytic cleavage and sulfation (to form the core protein of a chondroitin sulfate proteoglycan) contribute further to the heterogeneity of li chain types that are encountered.

It is known that chronic lymphocytic leukemia (CLL) cells express large amounts of li chain, and it has also been shown that neoplastic cells may show abnormalities in glycosaminoglycan synthesis. For these reasons, we felt that it would be of interest to study the li chain synthesis in peripheral blood (PB) lymphocytes from patients with CLL and to compare it with that in Epstein-Barr virus (EBV)–transformed normal PB lymphocytes (EBVL) and neoplastic Raji cells derived from a B lymphoma.

We have developed an IgG2a monoclonal antibody (MoAb), VCD-1, that reacts with an epitope on the N-terminal intracellular region of li. In this study, we report on experiments in which we have used VCD-1 to show that processing of li does not follow the same pattern in all class II positive cells and that CLL lymphocytes express an unusually large amount of the minor p35 form of li.

MATERIALS AND METHODS

Antibodies. VCD-1 MoAb reacts with an intracellular aminoterminal epitope on li. It was produced in this laboratory by fusion of SP2 myeloma cells with splenocytes from a Ballyc mouse that had been immunized with CLL lymphocytes. Biotinylated VCD-1 was prepared according to the method of Stahli et al. Sheep anti-mouse Ig antibody and monoclonal mouse anti-horse-radish peroxidase were produced in this laboratory.

Cells. The Raji cell line was obtained from American Type Culture Collection (Rockville, MD); the EBVL cell lines were established by transformation of normal PB lymphocytes with EBV from the supernatant of B95-8 cells. CLL cells were PB lymphocytes from patients with CLL and were separated by Ficoll-Hypaque flotation. Cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and antibiotics.

Reagents. The following reagents were obtained commercially from the indicated suppliers: formalin-fixed suspension of Staphylococcus aureus (SA); GIBCO-BRL (Grand Island, NY); sodium sulphate, H-thiouracil, Amplify, and Hyperfilm MP (Amersham, Arlington Heights, IL); endoglycosidase-H (endo-H) and long-arm bixin, Sigma (St. Louis, MO); Amphotelines, Pharmacon-LKB (Piscataway, NJ); urea, Schwarz-Mann (Orangeburg, NY); Immobilon P (polyvinylidene difluoride membrane), Millipore (Bedford, MA); and avidin/biotinylated peroxidase (ABC) reagent, Dako (Glostrup, Denmark). Peroxidase-antiperoxidase (PAP) reagent was produced in this laboratory; it contained monoclonal mouse antiperoxidase antibody (ascites at 1/100 dilution) and horseradish peroxidase (Serva, Cape Town, South Africa) at 100 μg/mL of phosphate-buffered saline (PBS).

Preparation of cell lysates. Cells were washed twice in PBS and
suspended in lysis buffer (PBS containing 0.5% Nonidet P-40 and 1 mmol/L phenylmethylsulphonyl fluoride (PMSF)) at a concentration of $5 \times 10^5$ cells/mL for B-lymphoblastoid cells and of $10^5$ cells/mL for CLL lymphocytes. After 20 to 30 minutes on ice, nuclei were pelleted at 1,000g; supernatants were stored at $-80^\circ$C until use.

**Western blotting.** Cell lysates were electrophoresed in polyacrylamide gels, in the presence of 2% sodium dodecyl sulphate (SDS-PAGE), using the Laemmli buffer system under nonreducing conditions. Blotting and labeling of the blot with VCD-1 was performed essentially according to De Blas and Cherwinski. Proteins were electro-blotted onto Immobilon P at 3.5 V/cm overnight. The membranes were blocked with 5% FCS in Tris-buffered saline and reacted sequentially with VCD-1 ascites (diluted 1/500 in blocking solution), with sheep antimouse IgG link antibody (whole sheep serum diluted 1/200), with PAP 1/20, with a second round of link antibody and PAP, and, finally, with diaminobenzidine (DAB) containing 0.03% cobaltous chloride.

**Metabolic labeling of cells with $^{35}$S-sulphate.** Sulphate-free medium was prepared by adding minimum essential essential medium (MEM) amino acid mixture (Flow Laboratories, Irvine, CA) and MEM vitamin mixture (International Scientific Industries, Cary, IL) to the salts of MEM with MgSO$_4$ replaced with 170.8 mg/L MgCl$_2$-6H$_2$O. Cells were washed twice in PBS and suspended at $10^5$ cells/mL in sulphate-free medium with 2% dialyzed FCS. After 30 minutes at 37°C, 30 μCi/mL of $^{35}$S-sodium sulphate was added; the cells were incubated overnight, washed, and lysed.  

**Pulse-chase labeling with $^{3}$H-leucine.** Cells were washed twice with PBS and suspended, at $2 \times 10^5$ cells/mL in leucine-free MEM containing 2% dialysed FCS and incubated for 30 minutes at 37°C.  

**Immunoprecipitation.** Formalin-fixed $S_A$ cells were washed twice in PBS containing 0.5% Nonidet P-40 + 2 mmol/L leucine + 0.02% azide ($S_A$ buffer) and resuspended at 10% in $S_A$ buffer containing 1 mg/mL ovalbumin. After 30 minutes on ice, 5 μL of VCD-1 ascites was added per 200 μL $S_A$ suspension. After 1 hour on ice, the $S_A$ were washed twice with $S_A$ buffer. For clearing lysates and for control immunoprecipitations, the $S_A$ were coated similarly with an irrelevant MoAb against $\beta$-galactosidase. Lysates were then cleared by incubating for 1 hour on ice, with $S_A$ coated with control antibody. The samples were centrifuged, and supernatants were immunoprecipitated with $S_A$ coated with VCD-1 for 1 hour. The $S_A$ were then washed 5 times with $S_A$ buffer, boiled with SDS-PAGE sample buffer (0.063 mol/L Tris-HCl pH 6.8, 10% wt/vol sucrose, 2.3% wt/vol SDS, and 0.002% bromophenol blue), and centrifuged. The supernatant solutions were loaded onto gels.

**RESULTS**

**VCD-1 immunoprecipitates all known monomeric forms of $\alpha_2$.** When EBV were incubated with $^{3}$H-leucine, lysed, and immunoprecipitated with VCD-1, two-dimensional electrophoresis and fluorography of the acrylamide gel (Fig 1) showed spots representing the p33, p35, and p41 forms of $\alpha_2^{123}$ as well as the p25 protolytic cleavage fragment. The most abundant form was that with molecular weight (M$_r$) 33 Kd (p33); p35, the product of translation from an alternative RNA initiation codon, and p41, which is encoded by the mRNA with alternatively spliced exons, were precipitated in lesser amounts.  

**CLL lymphocytes express a relatively large amount of the p35 form of $\alpha_2$.** In Western blots of EBV and Raji cell lysates, VCD-1 labeled a prominent band of the major p33 form of $\alpha_2$, the 66-Kd dimeric complexed form, and a faint band of M, 35 Kd that could have represented the p35 form or the sitidly, "processed" form of p33. However, in CLL lymphocytes, this higher M, form was abundant as the major p33 form (Fig 2A). When Western blots were performed with cell lysates of EBV from 5 different donors (Fig 2B) and of lymphocytes from 5 different EBV patients (Fig 2C), the same pattern was consistently observed. CLL lymphocytes contained more of the 35-Kd species, relative to the 33-Kd species, than do EBV.

To determine if the more abundant 35-Kd form in CLL cells represented p35 or the sialic acid-derivatized form of p33, we immunoprecipitated cell lysates with VCD-1 and separated the precipitated proteins by NPHEG and SDS-PAGE in two dimensions. These were then electrophoresed and detected with biotinylated VCD-1 and ABC reagent (Fig 3). Clearly, CLL lymphocytes expressed a large amount of the p35 form of $\alpha_2$ and very little processed p33 (Fig 3B); EBV (Fig 3A) and Raji cells (Fig 3C) produced only very small amounts of p35 and slightly more of the sialic acid-derivatized protein. Figure 3D shows an immunoblot with VCD-1 of CLL lystate precipitated with control antibody.

It is processed to form the core protein of significant amounts of chondroitin sulphate proteoglycan (CSPG) in Raji cells but not in EBV. When Raji cells and EBV were labeled with $^{35}$S-sulphate, lysed, immunoprecipitated with VCD-1, and analyzed by SDS-PAGE, the results, shown in Fig 4, were strikingly different. No CSPG could be precipitated from EBV (lane 4), whereas Raji cells had synthesized a polydisperse "smudge" of labeled protein.
ranging in M, from approximately 63 to 97 Kd (lane 3). The labeled, unprecipitated cell lysates were included in the gel (lane 1, Raji; lane 2, EBVL) to show that proteoglycans, although of different M, were produced by EBVL, but these did not bind to VCD-1. CLL cells produced no detectable CSPG (results not shown).

II is processed in the Golgi apparatus in Raji cells but only minimally so in EBVL. When Raji cells and EBVL were labeled with $^3$H-leucine, "chased" with nonradioactive leucine for varying times, and then lysed and immunoprecipitated with VCD-1, the immunoprecipitates were resolved into different patterns by SDS-PAGE. The fluorographs of the gels, run under nonreducing conditions, are shown in the left hand panel of Fig 5. In Raji cells, the immunoprecipitated II clearly increased in M, during the 2-hour chase, whereas, in the case of the EBVL, it was simply chased out of the pool without being modified. To see if this increase in pulse-chase pattern was due to processing in the Golgi apparatus, we treated VCD-1 immunoprecipitates of the chased Raji cells and EBVL with endo-H. Complex oligosaccharides generated in the Golgi apparatus are resistant to endo-H, whereas high mannose oligosaccharides generated in the ER are cleaved, resulting in lower M, forms. The fluorographs of endo-H-treated VCD-1 immunoprecipi-

tates of pulse-chased Raji cells and EBVL are shown in the right hand panels of Fig 5. In the Raji cells an endo-H-resistant band appeared after 30 to 60 minutes of chase; such a band was hardly detectable in EBVL. This difference in processed p33 is also shown in Fig 3A and C.

**DISCUSSION**

The results of these studies have shown that the MoAb VCD-1 precipitated the p33, p35, and p41 forms of the class II invariant chain and their sulphated and sialylated derivatives. Thus, the antibody could be used to estimate relative abundances of the different II species in lymphocyte populations representative of the transformed or neoplastic B-cell phenotype. It is from this application that two observations of interest emerged.

First, PB lymphocytes from patients with CLL were consistently found to contain larger amounts of the p35 form of the II chain relative to the p33 species. On the other hand, in the case of EBV-transformed normal B cells or Raji cells, a very faint 35-Kd band was seen, and p33 was the predominant species. Two-dimensional electrophoresis confirmed the identity of the 35-Kd band in CLL as p35.

Although CLL lymphocytes are known to express increased levels of II, this is, to the best of our knowledge, the
Fig 2. Western blots of CLL lymphocytes, EBVL, and Raji cells with VCD-1 MoAb. Lysates of lymphoid cells were subjected to SDS-PAGE in 11% acrylamide gels under nonreducing conditions. Separated proteins were electrophoretically transferred onto an Immobilon P membrane, and the membrane was reacted with VCD-1 MoAb, sheep-antimouse Ig link antibody, PAP, and DAB substrate as in Materials and Methods. (A) EBVL, lane 1; Raji, lane 2; and CLL, lane 3. (B) EBVL from 5 different donors. (C) CLL lymphocytes from 5 different patients. Arrows indicate positions and M, in kilodaltons, of marker proteins.

First report of the excessive accumulation in these cells of the b3 species whose translation is initiated from an AUG codon that is upstream from the p33 start codon. The abnormal p35:p33 ratios seen in all of the CLL cells that we examined suggests that this is a characteristic feature of this neoplastic disorder and, as such, merits further study. The fact that both p33 and p35 species are present, but in abnormal relative amounts, raises the possibility that mechanisms that control translation, and that have hitherto been characterized only in prokaryotic cells,27,28 may be deranged in CLL. The excessive accumulation of p35 in CLL cells is of interest because this molecule is known to have a strong N-terminal ER-retention signal16 and to form mixed trimers with p33. Therefore, in CLL cells it seems probable that both p35 and

Fig 3. Western blots of VCD-1 immunoprecipitates after separation by NEPHGE and SDS-PAGE. Unlabeled lysates of EBVL (A), CLL lymphocytes (B), and Raji cells (C) were immunoprecipitated with VCD-1, and the precipitated proteins were separated by NEPHGE and SDS-PAGE with the acidic end to the right as described in Materials and Methods. After electrophoretic transfer to Immobilon P membrane, the membranes were reacted with biotinylated VCD-1, ABC reagent, and DAB substrate. (D) A control blot, i.e., CLL lysate immunoprecipitated with control MoAb (anti-β-galactosidase) and reacted with biotinylated VCD-1 after NEPHGE, SDS-PAGE, and electrophoretic transfer. Arrows mark the spots representing the p35 form of b3. The significance of spots (i) and (ii) is not known.
p33 are retained in the ER without passing through the Golgi apparatus. This is consistent with our observation that glycosylation and sialylation of li chains was barely detectable in CLL cells.

In an elegant series of transfective experiments, Clements et al. were able to correlate li chain expression with tumorigenicity of Sal murine sarcoma cells. They suggested that overexpressed li chains blocked class II peptide-binding sites and so prevented presentation, via the class I route, of endogenous tumour-associated peptides. These observations, considered in conjunction with our findings, suggest that excessive li chain accumulation in CLL cells may contribute to the expression of the neoplastic phenotype by interfering with effective immune surveillance. Indirect support for this notion is to be found in a report by Sekaly et al. that showed class II molecules are able to present endogenous peptides in the absence of invariant chain.

Second, we have shown that Raji cells, derived originally from a malignant Burkitt lymphoma, differ from EBVL in that the malignant cells produced a CSPG with li as core protein and added endo-H-resistant complex oligosaccharides to their li chains, whereas the transformed cells did so to much less of an extent.

The significance of this finding is, as yet, uncertain. It may be related to the neoplastic nature of the cells, because it is known that intercellular glycosaminoglycans from mammalian tumour tissues differ significantly from those of the tissue of origin. Bono et al. have shown that M₆s of sulphated li chains isolated from malignant B cells are generally larger than those purified from normal B cells.

Furthermore, the in vitro killing of K562 target cells by natural killer cells is inhibited by complex carbohydrates; containing α(2 → 6)-sialic acid residues. Thus, the presence of such polysaccharides on the cell surfaces of B-lym-
Fig 5. Pulse-chase of II immunoprecipitated with VCD-1 and treated with endo-H. RAJ cells and EBVL were labeled for 10 minutes with 3H-leucine and chased for 0, 0.6, 1, and 2 hours in medium with nonradioactive leucine. Cells were lysed and immunoprecipitated with VCD-1. One-half of the SA with the immunoprecipitated proteins was treated with endo-H (right-hand panels), and the other half with buffer alone (left-hand panels) as in Materials and Methods. Proteins were solubilized in nonreducing sample buffer and separated by SDS-PAGE in a 11% acrylamide gel, which was treated with Amplify, dried, and fluorographed.

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Occasional Surveys

BONE-MARROW AUTOTRANSPLANTATION IN MAN

Report of An International Cooperative Study*

Summary Bone-marrow autotransplantation consists of the administration of extremely high doses of chemotherapy and/or radiation followed by “rescue” with autologous, cryopreserved, bone-marrow cells. This approach can produce responses unattainable with conventional doses of similar agents. Bone-marrow autotransplantation is relatively easy to do. This report summarizes data from 2570 patients receiving autotransplants at 43 centres worldwide for hematological malignancies and solid tumours; more than 50% of these transplants were done since 1984. Most transplants were performed for treatment of lymphoma, leukemia, lung cancer, melanoma, neuroblastoma, and breast cancer. Preliminary analyses indicate favorable responses in some tumour types and provide a basis for future investigations.

INTRODUCTION

Many types of tumours in man show a dose-response relation to antineoplastic drugs and radiation,1 but the amount that can be given is limited, primarily by toxic effects on the bone marrow. This limitation can now be circumvented by intravenous infusion of normal haemopoietic stem cells, either from the patient or a histocompatible donor. Infusion of the patient’s own bone marrow is referred to as an autologous bone-marrow transplant or autotransplant. Drug and/or radiation doses can be raised by 1.5-3 times when followed by an autotransplant.

There are two important considerations in selecting appropriate tumours for treatment in conjunction with bone-marrow autotransplantation. First, the tumour should show a dose-response relation to antineoplastic drugs or radiation. Secondly, the natural history of the tumour should be such that bone-marrow involvement is unlikely. Tumours that meet these criteria include acute leukemia, neuroblastoma, breast, ovarian, and testicular carcinoma, and certain lymphomas such as Hodgkin’s disease and aggressive non-Hodgkin lymphomas. In cases in which the bone marrow has been affected it may be possible to remove potentially contaminating tumour cells by immunological, pharmacological, or physical techniques.2-3 It may also be possible to use an alternative source of haemopoietic stem cells such as peripheral blood.4,5

There are considerable animal data to suggest that when the dose of drugs and/or radiation can be raised by doing bone-marrow autotransplantation a therapeutic response can be obtained in tumours resistant to conventional doses,6-7 and that the less extensive the disease, the better the response. Initial data in man indicate that high-dose chemotherapy, with or without radiation, followed by autotransplantation produces responses in patients whose tumours were resistant to conventional doses of these agents.8-10 Recent data suggest that response varies inversely with degree of spread,9-12 long-term, disease-free survival has been achieved in some instances.13,14

Bone-marrow autotransplantation is being used increasingly in man. There is no central registry of autotransplant data, as there is for allogeneic bone-marrow transplantation. Furthermore, much recent data is reported only at meetings or as abstracts. This report gives data from many centres active in bone-marrow autotransplantation. The objective of the study was to determine activity in this area and to obtain an indication of the types of tumours that respond to treatment with autotransplantation.

METHODS

Centres doing bone-marrow autotransplantation were identified by several means—for example, through international meetings or a review of publications indexed in the National Library of Medicine. Questionnaires were sent to 67 centres and answered by 43-15 centres, 24 (36%) in North America, and 4 (6%) elsewhere. Data were requested on 1-2 occasions to identify cases transplanted up to July, 1985.

Information was collected on number of new cases per year, in-vivo bone-marrow manipulation, cryopreservation, numbers of patients treated and tumour types, and drug and radiation regimens. Treatment outcome was classified as complete, partial, or less-than-partial response, by the use of standard criteria. Median duration of remission, duration of complete remission, and number of patients in continuous remission for more than 24 months were also ascertained. Data for January-July, 1985, were used to give a projection for rates for 1985.

RESULTS

Bone-marrow autotransplants were done in 2570 patients at the 43 centres. The annual and cumulative rates (fig 1)

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Professor Peter Jacobs
Fig 1—Annual and cumulative frequency of bone-marrow autotransplants done by the participants in this study.

Data for 1985 have been obtained by projection.

show that more than 50% of transplants have been done since 1984.

In most cases the bone marrow was not manipulated in vitro to remove potentially contaminating malignant cells.

22 centres (51%) did so on more than one occasion, usually by the use of monoclonal antibodies (12 centres) or drugs (10 centres). All centres used cryopreserved bone marrow most of the time. At 11 centres some patients received non-cryopreserved bone-marrow.

935 transplants (36%) were done for lymphomas, including Hodgkin's disease (155, 4%), Burkitt's lymphoma (76; 3%), and other non-Hodgkin lymphomas (744; 29%) (fig 2). The non-Hodgkin lymphomas were diffuse large-cell or immunoblastic lymphomas. 588 transplants (23%) were done for leukaemias, including acute myelogenous leukaemia (246; 10%), acute lymphoblastic leukaemia (279; 11%), and chronic myelogenous leukaemia (83; 3%), 41% (1047) were done for non-haematological solid tumours.

Fig 2—Tumour types in patients treated with bone-marrow autotransplantation.

Fig 3—Preliminary response rates, expressed as percentage of the total number of cases, in patients treated by bone-marrow autotransplantation.

Because of range of patient characteristics and cytoreductive regimen, these data should be interpreted with caution.

Partly because the patients included in this study had diverse tumours at different disease stages and were treated with a wide variety of drug and radiation protocols, it is difficult to determine response rates accurately. Preliminary overall response rates are indicated in fig 3. Prominent among the responsive tumours are lymphomas, leukaemias, Wilms' tumour, neuroblastoma, breast, and ovarian carcinoma. Less commonly responses were observed in gastrointestinal malignancies and non-small-cell lung cancer.

DISCUSSION

Our international survey indicates that bone-marrow autotransplantation is increasingly being done in patients with cancer, mainly in those with lymphomas and leukaemias, but recently also in patients with solid tumours. The annual rate of autotransplantation is increasing rapidly; 50% of all procedures have been done since 1984, and more than 1000 will probably be done in 1986 alone.

Considerable experimental data suggest that high-dose chemotherapy and/or radiation with autotransplantation can be effective. Preliminary results of phase I-II trials in man are encouraging. The dose of antineoplastic drugs and radiation can be 1-5 times higher with bone-marrow autotransplantation than without, and although most autotransplants have been done in patients with advanced cancer resistant to conventional doses of antineoplastic agents, favourable results have been observed. Laboratory data clearly indicate that the earlier in the disease that autotransplantation is done, the better the response. Preliminary data in man support this finding. Some patients with tumours such as aggressive lymphomas that do not respond to chemotherapy and radiation can now be cured with high-dose chemotherapy and bone-marrow autotransplantation. The proportion of such patients who could be cured might be increased if transplants were done earlier.

Our data indicate certain types of tumours likely to respond to autotransplantation and high-dose chemotherapy/radiation. Prominent among these are lymphomas, leukaemias, breast and ovarian cancers, and neuroblastomas. In these instances, phase III trials would
be appropriate. Other tumours, such as gastrointestinal malignancies and non-small-cell lung cancer, seem less responsive. Yet other tumours have not yet been thoroughly investigated.

The response rates presented in this paper ought to be interpreted with caution since data are from heterogeneous groups of patients in different disease stages receiving different forms of pretransplant chemotherapy and/or radiation cytoreductive regimens. However, high-dose chemotherapy and radiation combined with bone-marrow autotransplantation seems to be a promising form of treatment for patients with advanced cancer in whom conventional approaches are unlikely to be successful.

Correspondence should be addressed to the Advisory Committee for the International Autologous Bone Marrow Transplant Registry, c/o Dr J. O. Amstutz, Department of Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68105, USA or R. G. O., Department of Medicine, UCLA School of Medicine, Los Angeles, California 90095, USA.

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References continued at foot of next column.
Increasing Utilization of Allogeneic Bone Marrow Transplantation
Results of the 1988-1990 Survey

Mortimer M. Bortin, MD; Mary M. Horowitz, MD; and Alfred A. Rimm, PhD

Objective: To determine the pattern and frequency of allogeneic bone marrow transplantation from related and unrelated donors from 1988 to 1990.

Design and Setting: Survey of 342 institutions in 47 countries.

Measurements: Numbers of patients receiving bone marrow transplantation for specific disease categories at institutions with active allogeneic bone marrow transplant programs.

Main Results: Patients (14,745) received allogeneic bone marrow transplantation between 1988 and 1990; of these, 11,531 (8%) were from unrelated donors. Reasons for transplantation were acute leukemia (47%), chronic myelogenous leukemia (27%), lymphoma and other malignancies (10%), severe aplastic anemia (9%), and other nonmalignant diseases (7%). The number of allogeneic bone marrow transplants per million persons differed among countries, averaging 7.7 per million in North America and 5.7 per million in western Europe.

Conclusions: The use of allogeneic bone marrow transplantation continued to increase at a rate of more than 600 additional patients and 25 new transplant teams annually. This rise is due in part to increasing use of unrelated volunteers as donors. Resources allocated for transplants vary widely among countries.

Methods

We used the IBMTR Directory of Bone Marrow Transplant Teams (3) to identify all institutions at which allogeneic bone marrow transplantation was done. Several mechanisms ensure that all allogeneic transplant teams are included in this directory. First, most teams participate in the IBMTR research program and submit detailed information to the IBMTR Statistical Center regarding their consecutive patients receiving bone marrow transplantation. Second, the biomedical literature is screened monthly for articles on clinical bone marrow transplantation using the U.S. National Library of Medicine's MEDLINE to identify institutions not listed in the directory. Third, the IBMTR maintains a booth at meetings of various organizations including the American Society of Clinical Oncology, American Society of Hematology, International Society for Experimental Hematology, International Society of Hematology, and the UCLA Symposia on Bone Marrow Transplantation, where physicians who do bone marrow transplantation are encouraged to identify themselves and their institutions. Fourth, we reviewed a list of all teams submitting data to the European Bone Marrow Transplant Group (4) for 1990. Finally, members of the IBMTR Advisory Committee, representing 15 countries including the geographic areas where most bone marrow transplants are done, help identify institutions not found previously.

We identified 448 institutions in 52 countries in which allogeneic bone marrow transplantation was known or believed to have been done between 1988 and 1990. Although this list is complete to the best of our knowledge, allogeneic transplants may have been done elsewhere. Any such omissions are unintentional.

Questionnaires were mailed to leaders of transplant teams or heads of hematology-oncology departments at all 448 institutions. A duplicate questionnaire was mailed 2 months later to nonrespondents. Those not replying 2 months after the second mailing were contacted by telephone, telefax, or express mail.

Questionnaires requested information regarding the numbers of patients receiving syngeneic, allogeneic-related, allogeneic-unrelated, and autologous bone marrow transplantation for specific disease categories in 1988, 1989, and 1990. The category of acute leukemia included acute lymphoblastic and acute myelogenous leukemia plus a small number of patients with hybrid or dual-lineage leukemia. The category of chronic leukemia included primarily chronic myelogenous leukemia plus a small number of patients with chronic lymphocytic leukemia. Malignant lymphoma included non-Hodgkin and Hodgkin lymphomas. Other malignancies included myelodysplastic syndromes, multiple myeloma, and a variety of solid tumors.

Institutional data were requested including total number of beds, number of beds assigned for patients in the hematology-oncology unit, number of beds for patients having bone marrow transplantation, and the year that bone marrow transplants were first done.

Results

Response Rates to Questionnaire

Completed questionnaires were returned by physicians at 418 of the 448 (93%) institutions surveyed.


From the International Bone Marrow Transplant Registry and the Medical College of Wisconsin, Milwaukee, Wisconsin. For current author addresses, see end of text.

The International Bone Marrow Transplant Registry (IBMTR) collects, organizes, and analyzes data on allogeneic and syngeneic (but not autologous) bone marrow transplantation. The IBMTR is a voluntary, multi-institutional group conducting research aimed primarily at determining the factors that affect the success and failure of bone marrow transplants. In addition to these clinical epidemiologic investigations, the IBMTR conducts periodic surveys to assess the use of allogeneic and syngeneic bone marrow transplantation worldwide (1, 2). Previous surveys have covered the period from 1955 to 1987; we report continuing increases in the use of allogeneic bone marrow transplantation from 1988 to 1990.
Table 1. Distribution of Patients Receiving Allogeneic or Syngeneic Bone Marrow Transplants between 1988 and 1990 by Type of Donor and Disease Category

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<th>Chronic Leukemia</th>
<th>Lymphoma</th>
<th>Other Malignancy</th>
<th>Aplastic Anemia</th>
<th>Thalassemia Major</th>
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<tr>
<td>Subtotal</td>
<td>366</td>
<td>510</td>
<td>10</td>
<td>50</td>
<td>97</td>
<td>1</td>
<td>41</td>
<td>50</td>
<td>28</td>
<td>1153</td>
</tr>
<tr>
<td>Total</td>
<td>2099</td>
<td>1095</td>
<td>199</td>
<td>217</td>
<td>383</td>
<td>44</td>
<td>131</td>
<td>61</td>
<td>71</td>
<td>4300</td>
</tr>
<tr>
<td>1988</td>
<td>2247</td>
<td>1313</td>
<td>239</td>
<td>253</td>
<td>429</td>
<td>145</td>
<td>127</td>
<td>89</td>
<td>74</td>
<td>4916</td>
</tr>
<tr>
<td>1990</td>
<td>2545</td>
<td>1502</td>
<td>261</td>
<td>310</td>
<td>446</td>
<td>150</td>
<td>128</td>
<td>93</td>
<td>95</td>
<td>5529</td>
</tr>
<tr>
<td>Total</td>
<td>6891</td>
<td>3910</td>
<td>698</td>
<td>780</td>
<td>1258</td>
<td>339</td>
<td>386</td>
<td>243</td>
<td>240</td>
<td>14745</td>
</tr>
</tbody>
</table>

Repeated attempts to establish contact with physicians at 30 (7%) institutions were unsuccessful. We assumed that most of these nonrespondents did few or no allografts between 1988 and 1990. We learned, however, that two teams with allograft programs did not respond because they did not wish to participate in the survey. From other sources (4) we determined that neither team did more than six allografts in 1990. We are confident that our study included all allograft programs worldwide with ≥ 30 patients annually.

No bone marrow transplants were done between 1988 and 1990 at 18 of the 418 institutions from which we obtained completed questionnaires. Physicians at most of these institutions reported that their transplant programs would begin in 1991 or later, whereas a few reported that their transplant programs were discontinued before 1988. Autologous, but not allogeneic, bone marrow transplantation was done at 58 of the 400 remaining institutions. Data from these institutions were excluded from our analysis. Of the responding institutions, 342 had allograft programs (Appendix 2). Data provided by team leaders at these 342 institutions formed the basis for our analysis.

Characteristics of Institutions

The median number of hospital beds at the 342 institutions where allografts were done between 1988 and 1990 was 600 (range, 50 to 3900 beds). The median number of beds specifically assigned for the care of patients in the hematology-oncology unit was 24 (range, 0 to 500 beds), and the median number of beds designated specifically for patients having bone marrow transplantation was 5 (range, 0 to 60 beds). As of 1990, the total number of beds designated for patients receiving bone marrow transplantation at these institutions was 2212. Of these 2212 institutions, 599 were in North America, 844 were in western Europe, and 373 were elsewhere.

Allogeneic and Syngeneic Transplants

Between 1988 and 1990, 14,745 patients received allogeneic or syngeneic bone marrow transplants at these 342 institutions: 4300 in 1988, 4916 in 1989, and 5529 in 1990. Based on data reported to the IBMTR from 1968 to 1990, we estimated that fewer than 2% of the donors and recipients were genetically identical twins. Unrelated donors were used for 1153 (8%) of patients: 212 (5%) in 1988, 392 (8%) in 1989, and 549 (10%) in 1990. At least one transplant from an unrelated donor was done at 167 institutions. The distribution of patients according to donor status (related or unrelated) and disease category is shown in Table 1. Eighty-three percent of transplants were done to treat malignant diseases and 17% to treat nonmalignant diseases.

Table 2. Geographic Distribution of Patients Receiving Allogeneic or Syngeneic Bone Marrow Transplants between 1988 and 1990 by Disease Category

<table>
<thead>
<tr>
<th>Area</th>
<th>Acute Leukemia</th>
<th>Chronic Leukemia</th>
<th>Lymphoma</th>
<th>Other Malignancy</th>
<th>Aplastic Anemia</th>
<th>Thalassemia Major</th>
<th>Immune Deficiency</th>
<th>Genetic-Metabolic</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>North America</td>
<td>2846</td>
<td>1779</td>
<td>378</td>
<td>363</td>
<td>464</td>
<td>16</td>
<td>166</td>
<td>102</td>
<td>120</td>
<td>6234</td>
</tr>
<tr>
<td>Western Europe</td>
<td>2920</td>
<td>1495</td>
<td>265</td>
<td>348</td>
<td>433</td>
<td>276</td>
<td>187</td>
<td>116</td>
<td>87</td>
<td>6137</td>
</tr>
<tr>
<td>Elsewhere</td>
<td>1115</td>
<td>636</td>
<td>35</td>
<td>69</td>
<td>361</td>
<td>47</td>
<td>33</td>
<td>25</td>
<td>33</td>
<td>2374</td>
</tr>
<tr>
<td>Total</td>
<td>6891</td>
<td>3910</td>
<td>698</td>
<td>780</td>
<td>1258</td>
<td>339</td>
<td>386</td>
<td>243</td>
<td>240</td>
<td>14745</td>
</tr>
</tbody>
</table>

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Table 2 shows the geographic distribution of patients by disease category. Most transplants were done in North America (42%) and western Europe (42%). The remainder were done in Asia (7%), Australia and New Zealand (3%), the Mideast and Africa (2%), South and Central America (2%), or eastern Europe (including the U.S.S.R.) (1%).

Based on world population approximations for 1989 (5), we estimated that between 1988 and 1990 the number of allogeneic transplants per million persons annually was about 7.7 in North America and 5.7 in western Europe. The distribution of allogeneic bone marrow transplantation procedures by country is shown in Table 3.

Between 1988 and 1990, 35 institutions did an average of one or fewer allogeneic transplants per year. At 120 (35%) of the 342 institutions, five or fewer patients had allogeneic transplants annually, accounting for 6% (822) of the 14,745 patients. In contrast, an average of 50 or more patients had allogeneic bone marrow transplantation per year at 16 (5%) of the 342 institutions and accounted for 25% (3,714) of the patients.

Figure 1 shows the annual and cumulative numbers and percentages of patients treated with allogeneic or syngeneic transplantation from 1 January 1981 through 31 December 1990. The increases during this 10-year period were linear and averaged 495 additional patients each year (least squares regression). Between 1988 and 1990, however, the increase averaged 615 patients per year. Based on the results of the current survey and previous IBMTR surveys (1, 2), we estimated that the cumulative total number of allogeneic and syngeneic transplant procedures was at least 14,745 between 1988 and 1990, 32,582 between 1981 and 1990, and 35,077 from the mid-1970s through 1990.

Of the 342 institutions with active allogeneic transplant programs between 1988 and 1990, 76 (22%) started between 1988 and 1990, 170 (50%) between 1981 and 1987, and 96 (28%) between 1960 and 1980.

### Autologous Bone Marrow Transplantation

We did not conduct a survey of worldwide autotransplant activity; results of such surveys have been previously reported (6, 7). However, to obtain an estimate of the total size of their bone marrow transplantation programs, physicians at the 342 institutions with autotransplant programs were also asked to provide data about their autotransplant activity. Only autologous bone marrow transplants were done between 1988 and 1990 at 41 of the surveyed institutions. At the 301 institutions with both autotransplant and allogeneic transplant programs, 14,187 patients received autotransplants, and 13,139 received autotransplants. These data yielded an overall ratio of 1.08:1.00; this ratio varied widely among institutions.

The number of patients receiving autologous bone marrow transplantation by disease category is shown in Table 4. The number of patients who received autotransplants at these institutions (where autotransplants were also done) increased more rapidly than did the number of patients receiving autotransplants between 1988 and 1990: 3,506 patients in 1988, 4,479 in 1989, and 5,154 in 1990. The increase averaged 824 additional patients per year.

<table>
<thead>
<tr>
<th>Country</th>
<th>Transplants per million</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>7.8</td>
</tr>
<tr>
<td>Australia</td>
<td>5.1</td>
</tr>
<tr>
<td>Austria</td>
<td>6.1</td>
</tr>
<tr>
<td>Belgium</td>
<td>0.5</td>
</tr>
<tr>
<td>Brazil</td>
<td>9.1</td>
</tr>
<tr>
<td>Canada</td>
<td>0.2</td>
</tr>
<tr>
<td>Chile</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>China</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Colombia</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Cuba</td>
<td>0.6</td>
</tr>
<tr>
<td>Czechoslovakia</td>
<td>5.9</td>
</tr>
<tr>
<td>Denmark</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Egypt</td>
<td>6.3</td>
</tr>
<tr>
<td>Finland</td>
<td>8.9</td>
</tr>
<tr>
<td>France</td>
<td>4.0</td>
</tr>
<tr>
<td>Germany</td>
<td>1.0</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>0.9</td>
</tr>
<tr>
<td>Hungary</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>India</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Indonesia</td>
<td>5.0</td>
</tr>
<tr>
<td>Ireland</td>
<td>10.3</td>
</tr>
<tr>
<td>Israel</td>
<td>5.3</td>
</tr>
<tr>
<td>Italy</td>
<td>2.2</td>
</tr>
<tr>
<td>Japan</td>
<td>0.3</td>
</tr>
<tr>
<td>Korea</td>
<td>0.3</td>
</tr>
<tr>
<td>Malaysia</td>
<td>6.2</td>
</tr>
<tr>
<td>Netherlands</td>
<td>6.4</td>
</tr>
<tr>
<td>New Zealand</td>
<td>1.4</td>
</tr>
<tr>
<td>Norway</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Philippines</td>
<td>0.2</td>
</tr>
<tr>
<td>Poland</td>
<td>2.8</td>
</tr>
<tr>
<td>Portugal</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Russia</td>
<td>3.6</td>
</tr>
<tr>
<td>Saudi Arabia</td>
<td>0.5</td>
</tr>
<tr>
<td>South Africa</td>
<td>4.6</td>
</tr>
<tr>
<td>Spain</td>
<td>6.4</td>
</tr>
<tr>
<td>Sweden</td>
<td>6.8</td>
</tr>
<tr>
<td>Switzerland</td>
<td>1.2</td>
</tr>
<tr>
<td>Taiwan</td>
<td>0.1</td>
</tr>
<tr>
<td>Thailand</td>
<td>0.2</td>
</tr>
<tr>
<td>Turkey</td>
<td>6.2</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>7.5</td>
</tr>
<tr>
<td>U.S.A.</td>
<td>1.2</td>
</tr>
<tr>
<td>Uruguay</td>
<td>0.2</td>
</tr>
<tr>
<td>Venezuela</td>
<td>1.2</td>
</tr>
</tbody>
</table>

The geographic distribution of autotransplants by disease category is shown in Table 5. Most autotransplants were done in North America (50%) and western Europe (42%). The rest were done in Australia and New Zealand (3%), Asia (2%), the Mideast and Africa (2%), eastern Europe (including the U.S.S.R.) (<1%), and South and Central America (<1%).

Of the 301 institutions with both autotransplant and allogeneic transplant programs, 76 (26%) autotransplant programs began between 1988 and 1990, 176 (58%) between 1981 and 1987, and 48 (16%) between 1960 and 1980.

### Discussion

The use of allogeneic bone marrow transplantation increased at a rate of 615 additional patients per year.

It is now generally accepted that allogeneic bone marrow transplantation is superior to alternative therapies and offers the potential for cure in conditions such as the Wiskott-Aldrich syndrome (8), severe combined immunodeficiency disease (9), severe aplastic anemia (10), thalassemia major (11), and chronic myelogenous leukemia (12, 13). Although effective and widely used in the management of other diseases, such as the acute leukemias, its relative value in comparison with other approaches is less clear (14).

The increasing use of allotransplants may also be a result of increasing safety of the procedure. Recent improvements are reported in the prevention of graft-versus-host disease (15) and in the prevention and treatment of cytomegalovirus interstitial pneumonia (16, 17) (two major causes of treatment failure after allogeneic bone marrow transplantation).

One of the factors limiting more widespread use of allotransplants is the knowledge that results are generally inferior when a family member other than an HLA-

identical sibling is used as the donor (18, 19). Most patients (60% to 70%) who could benefit from a transplant do not have an HLA-identical sibling (20). Results much like those obtained with HLA-identical sibling donor transplants were recently reported using unrelated donors closely matched with recipients for HLA antigens (21-23). This finding has led to the development of large registries of persons who have volunteered to serve as bone marrow donors (24-26). The increasing use of unrelated donors (5% of all allografts in 1988, 8% in 1989, and 10% in 1990) accounts, in part, for the continuing increase in the use of allogeneic bone marrow transplantation.

Between 1988 and 1990 more allotransplants (14 187) than autotransplants (13 139) were done at the 301 institutions that did both types of transplantation. The rate of increase in autotransplants (824 per year), however, was higher than that of allotransplants (566 per year). Also, a more rapid increase occurred in new autotransplant programs compared with new allotransplant programs. Although we did not attempt to obtain data regarding all autotransplant programs worldwide, we are confident that the total number of patients having autologous bone marrow transplantation and the total number of institutions in which this procedure is done now exceed those of allografts.

Confirming a previous report (6), we found that more patients with lymphoma had transplantation at centers in North America than in western Europe and that more patients with acute leukemia had transplantation in western Europe than in North America. The reasons for these differences are not known.

The effect of center size on transplant outcome is an important issue for health care policy managers. A recent study from the IBMTR found that the number of allotransplants done to treat early leukemia annually at institutions was predictive of outcome (27). The adjusted probability of treatment-related mortality was higher (relative risk, 1.53; \( P < 0.01 \)) among patients having transplantation at centers in which five or fewer transplants were done per year than among those having transplantation at larger centers. It is therefore of some concern that 120 institutions in our survey averaged five or fewer allotransplants per year. Further study that takes into account other factors, such as annual number of autotransplants also done at these institutions, is needed to evaluate the influence of center size on outcome of allogeneic bone marrow transplantation.

Our data on the use of allotransplants in terms of

![Graph showing annual numbers of patients receiving allogeneic or syngeneic bone marrow transplants, 1981-1990. The data for 1981-1987 are from previous reports (1, 2), and the data for 1988-1990 are from this study.](image)

Table 4. Distribution of Patients Receiving Autotransplants at Institutions with Allotransplant Programs in 1988-1990 by Disease Category

<table>
<thead>
<tr>
<th>Year</th>
<th>Acute Leukemia</th>
<th>Chronic Leukemia</th>
<th>Lymphoma</th>
<th>Breast Cancer</th>
<th>Neuroblastoma</th>
<th>Other Solid Tumor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1988</td>
<td>1038</td>
<td>74</td>
<td>1327</td>
<td>229</td>
<td>229</td>
<td>609</td>
<td>3506</td>
</tr>
<tr>
<td>1989</td>
<td>1294</td>
<td>81</td>
<td>1760</td>
<td>336</td>
<td>260</td>
<td>748</td>
<td>4479</td>
</tr>
<tr>
<td>1990</td>
<td>1284</td>
<td>131</td>
<td>2219</td>
<td>452</td>
<td>232</td>
<td>836</td>
<td>5154</td>
</tr>
<tr>
<td>Total</td>
<td>3616</td>
<td>286</td>
<td>5306</td>
<td>1017</td>
<td>721</td>
<td>2193</td>
<td>13139</td>
</tr>
</tbody>
</table>

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national populations is, to our knowledge, the first analysis of this kind. It is not surprising that this expensive, high-technology procedure is available in fewer than 50 countries. In those countries without an allotransplant program, patients who might benefit either do not have transplantation or are referred elsewhere. The estimates of annual transplants per million persons in individual countries do not take into account the possible referral of patients across national borders. Although the United States has the largest number of patients receiving allotransplants (5552 between 1988 and 1990) and 87 institutions in which the procedure is done, 16 of 50 states do not have an allotransplant program. Here, also, patients who might benefit either do not have transplantation or are referred elsewhere.

It is impossible to assess the impact that the 1990 Nobel prize award to Dr. E. D. Thomas of Seattle for his pioneering work in clinical bone marrow transplantation will have on the field's future growth. Little doubt exists, however, that the expert training programs for young physicians at large transplant centers will lead to further increases in new transplant teams and in the number of patients offered this exciting new therapy.

Appendix 1. Members of the International Bone Marrow Transplant Registry Advisory Committee

This 96th report from the IBMTR was prepared for the Advisory Committee, IBMTR Advisory Committee: Robert Feller, MD, Chair, University of California, Los Angeles, California; Robert C. Ash, MD, Medical College of Wisconsin, Milwaukee, Wisconsin; Kerry Atkinson, MD, St. Vincent's Hospital, Darlinghurst, Australia; Fritz H. Bach, MD, University of Minnesota, Minneapolis, Minnesota; John Barrett, MD, MRC Path, The Royal Postgraduate Medical School, London, England; Dirk W. van Bekkum, MD, PhD, Radiobiological Institute TNO, Rijswijk, the Netherlands; James C. Biggs, MD, PhD, St. Vincent's Hospital, Darlinghurst, Australia; Karl G. Blume, MD, Stanford University Hospital, Stanford, California; Martiner M. Butlin, MD, Medical College of Wisconsin, Milwaukee, Wisconsin; Richard E. Champlin, MD, M. D. Anderson Cancer Center, Houston, Texas; Karel A. Dicke, MD, PhD, University of Nebraska Medical Center, Omaha, Nebraska; G. Ehninger, MD, Medicinal Universitatsklinik, Tubingen, Germany; Alain Fischer, MD, Hopital Necker Enfants-Malades, Paris, France; James Gajewski, MD, UCLA Center for Health Sciences, Los Angeles, California; Edmund Gehan, PhD, M. D. Anderson Cancer Center, Houston, Texas; Eliane Gluckman, MD, Hopital Saint-Louis, Paris, France; John M. Goldman, MD, Hammersmith Hospital, London, England; Robert A. Grand, MD, PhD, All Children's Hospital, St. Petersburg, Florida; Werner Heilbr, MD, Karl Marx University, Leipzig, Germany; P. Jean Hensel-Downey, MD, University of Kentucky Medical Center, Kentucky; Roger H. Herzig, MD, University of Louisville, Louisville, Kentucky; Wolfgang Hinterberger, MD, Medizinische Universitatsklinik, Vienna, Austria; Richard Hong, MD, University of Wisconsin Hospital & Clinics, Madison, Wisconsin; Mary M. Horowitz, MD, Medical College of Wisconsin, Milwaukee, Wisconsin; Niels Jacobsen, MD, Rigshospitalet, Copenhagen, Denmark; John H. Kersey, MD, University of Minnesota, Minneapolis, Minnesota; Hans-Jochem Kohl, MD, Universitat Muenchen, Munich, Germany; Bernard Kuhneck, MD, Universitat Ulm, Ulm, Germany, Alberto M. Marmont, MD, Ospedale San Martino, Genoa, Italy; Tohru Masuzaki, MD, Center for Adult Diseases, Osaka, Japan; Hans A. Messner, MD, PhD, Ontario Cancer Institute, Toronto, Canada; Carole Miller, MD, Johns Hopkins Oncology Center, Baltimore, Maryland; Richard J. O'Reilly, MD, Memorial Sloan-Kettering Cancer Center, New York, New York; Ricardo Pasquini, MD, Hospital de Clinicas, Curitiba, Brazil; Gordon Phillips, MD, University of British Columbia, Vancouver, Canada; Ray L. Powles, MD, Royal Marsden Hospital, Surrey, England; I. Grant Princte, MD, Royal Free Hospital, London, England; Josy Reiffers, MD, Groupe Hospitaliers du Haut Leveque, Pessac, France; Alfred A. Rimm, PhD, Medical College of Wisconsin, Milwaukee, Wisconsin; Olle Ringdén, MD, PhD, Huddinge University Hospital, Huddinge, Sweden; Jerome Ritz, MD, Dana-Farber Cancer Institute, Boston, Massachusetts; Jon J. van Rood, MD, PhD, University Hospital, Leiden, the Netherlands; Giri Rozman, MD, Universidad de Barcelona, Barcelona, Spain; Monika W. Schaefer, MD, University of Essen, Essen, Germany; Bruno Speck, MD, Med Universitatsklinik Kantonssspital Basel, Basel, Switzerland; Sante Tura, MD, Universita Degli Studi di Bologna, Bologna, Italy; Roy S. Weiner, MD, University of Florida, Gainesville, Florida.

Appendix 2. Roster of Institutions Performing at Least One Allogeneic Bone Marrow Transplant between 1988 to 1990 by Geographic Region

Argentina

British Hospital of Buenos Aires, Buenos Aires; Clinica Independencia, Buenos Aires; Navy Hospital Pedro Mello, Buenos Aires; Hospital Privado de Cordoba, Cordoba.

Australia

Institute of Medical and Veterinary Science, Adelaide; Royal Brisbane Hospital, Brisbane; Royal Alexandria Hospital for Children, Camperdown; Royal Prince Alfred Hospital, Camperdown; St. Vincent's Hospital, Darlinghurst; Royal Hobart Hospital, Hobart; Royal Melbourne Hospital, Melbourne; Royal Children's Hospital, Parkville; Royal Perth Hospital, Perth; Alfred Hospital, Prahran; Prince of Wales Children's Hospital, Randwick; Royal North Shore Hospital, Sydney; Westmead Hospital, Sydney; Queen Elizabeth Hospital, Woodville.

Austria

University Children's Hospital, Graz; University Hospital, Innsbruck; St. Anna Children's Hospital, Vienna; Universitatsklinik Vienna, Vienna.
Kanagawa Cancer Center, Yokohama; Kanagawa Children’s Medical Center, Yokohama.

Korea
Catholic University Medical College, Seoul; Seoul National University Hospital, Seoul.

Malaysia
University of Malaya, Kuala Lumpur.

The Netherlands
Leiden University Hospital, Leiden; University Hospital, Maastricht; University of Nijmegen, Nijmegen; Dr. Daniel den Hoed Cancer Center, Rotterdam; University Hospital, Utrecht.

New Zealand
Auckland Hospital, Auckland; Christchurch Hospital, Christchurch; Wellington Hospital, Wellington.

Norway
Rikshospitalet, Oslo.

Philippines
National Kidney Institute, Quezon City.

Poland
Institute of Pediatrics, Poznan; Central Clinical Hospital, Warsaw; Ludwik Hirsfeld Institute of Immunology, Wroclaw.

Portugal
Hospital de Santa Maria, Lisbon; Instituto Portugues de Oncologia Francisco Gentil, Lisbon.

Russia
Clinical Hospital No. 6, Moscow.

Saudi Arabia
King Faisal Specialist Hospital & Research Centre, Riyadh; Riyadh Armed Forces Hospital, Riyadh.

South Africa
University of Cape Town, Cape Town; University of Witwatersrand Medical School, Johannesburg.

Spain
Hospital Sant Pau, Barcelona; Hospital Vall d’Hebron, Barcelona; University of Barcelona, Barcelona; Hospital Regional Reina Sofia, Cordoba; Hospital Ramon y Cajal, Madrid; Hospital de la Princesa, Madrid; Clinica Puerta de Hierro, Madrid; Hospital Carlos Haya, Malaga; Hospital Ntra. Sra de Aranzazu, San Sebastian; Hospital Marques de Valdecilla, Santander; Hospital Universitario Le Fe, Valencia.

Sweden
University of Goteborg, Goteborg; Huddinge University Hospital, Huddinge; University Hospital, Lund; University Hospital, Uppsala.

Switzerland
Kantonsspital, Basel; University Hospital, Geneva; Kantonsspital, Zurich.

Taiwan
National Taiwan University Hospital, Taipei; Veterans General Hospital, Taipei.

Thailand
Siriraj Hospital, Bangkok.

Turkey
Gulhane Military Medical Academy, Ankara; University of Ankara, Ankara.

United Kingdom
East Birmingham Hospital, Birmingham; Queen Elizabeth Hospital, Birmingham; Royal Victoria Hospital, Bournemouth; Addenbrooke’s Hospital, Cambridge; University Hospital of Wales, Cardiff; Royal Infirmary, Edinburgh; Royal Devon & Exeter Hospital, Exeter; Royal Infirmary, Glasgow; Royal Hospital for Sick Children, Glasgow; St. James’s University Hospital, Leeds; Royal Infirmary, Leicester; Royal Hospital, Liverpool; Charing Cross Hospital, London; Hammersmith Hospital, London; Hospital For Sick Children, London; Institute of Child Health, London; Royal Free Hospital, London; Royal Marsden Hospital, London; St. George Hospital Medical School, London; Tenbrookton Clinic, London; The London Hospital, Whitechapel, London; University College Hospital, London; Westminster Children’s Hospital, London; Christie Hospital, Manchester; Royal Infirmary, Manchester; Royal Children’s Hospital, Manchester; Northwick Park Hospital, Middlesex; Royal Victoria Infirmary, Newcastle Upon Tyne; City Hospital, Nottingham; John Radcliffe Hospital, Oxford; Derriford Hospital, Plymouth.

United States
Arkansas Cancer Research Center, Little Rock, AR; Arkansas Children’s Hospital, Little Rock, AR; University of Arizona Cancer Center, Tucson, AZ; Alta Bates Herrick Hospital, Berkeley, CA; City of Hope National Medical Center, Duarte, CA; Scripps Clinic and Research Foundation, La Jolla, CA; Cedars-Sinai Medical Center, Los Angeles, CA; Children’s Hospital, Los Angeles, Los Angeles, CA; Southern California Permanente Medical Group, Los Angeles, CA; UCLA Center for Health Sciences, Los Angeles, CA; Children’s Hospital of Orange County, Orange, CA; St. Joseph’s Hospital Regional Cancer Center, Orange, CA; Children’s Hospital, San Diego, CA; Pacific Presbyterian Medical Center, San Francisco, CA; University of California San Francisco, San Francisco, CA; Stanford University Hospital, Stanford, CA; University of Colorado, Denver, CO; University of Connecticut Health Center, Farmington, CT; Yale University School of Medicine, New Haven, CT; Children’s Hospital, Washington, DC; George Washington University Medical Center, Washington, DC; Georgetown University Medical Center, Washington, DC; Medical Center of Delaware, Newark, DE; University of Florida, Gainesville, FL; All Children’s Hospital, St. Petersburg, FL; H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL; Emory University, Atlanta, GA; St. Francis Medical Center, Honolulu, HI; University of Iowa Hospitals and Clinics, Iowa City, IA; Humana Medical Reece Hospital, Chicago, IL; Rush Presbyterian—St. Luke’s Medical Center, Chicago, IL; University of Chicago, Chicago, IL; Loyola University Medical Center, Maywood, IL; Indiana University Hospital, Indianapolis, IN; Methodist Hospital of Indiana, Indianapolis, IN; University of Kansas Medical Center, Kansas City, KS; Cancer Center of Kansas, Wichita, KS; University of Kentucky, Lexington, KY; James Graham Brown Cancer Center, Louisville, KY; Louisiana State University Medical Center, New Orleans, LA; Brigham & Women’s and Children’s Hospital, Boston, MA; Dana-Farber Cancer Institute, Boston, MA; Johns Hopkins Oncology Center, Baltimore, MD; University of Michigan, Ann Arbor, MI; Wayne State University, Detroit, MI; University of Minnesota, Minneapolis, MN; Mayo Clinic, Rochester, MN; Washington University Medical Center, St. Louis, MO; Duke University Medical Center, Durham, NC; North Carolina Baptist Hospital, Winston-Salem, NC; Bishop Clarkson Memorial Hospital, Omaha, NE; University of Nebraska Medical Center, Omaha, NE; St. Joseph’s Hospital & Medical Center, Phoenix, NE; Montefiore Medical Center, Bronx, NY; North Shore Universi—
sity Hospital, Mansfield, NJ; Memorial Sloan Kettering Cancer Center, New York, NY; Mt. Sinai Medical Center, New York, NY; Sinai Memorial Hospital, Rochester, NY; New York Medical College, Valhalla, NY; University of Cincinnati, Cincinnati, OH; Cleveland Clinic, Cleveland, OH; University of Cleveland, Cleveland, OH; Ohio State University, Columbus, OH; University of Oklahoma, Oklahoma City, OK; Hah- nemann University, Philadelphia, PA; Temple University Comprehensive Cancer Center, Philadelphia, PA; University of Pennsylvania, Philadelphia, PA; Montefiore University Hospital, Pittsburgh, PA; Medical University of South Carolina, Charleston, SC; University of Tennessee Medical Center, Knoxville, TN; St. Jude Children's Research Hospital, Memphis, TN; Vanderbilt University Medical Center, Nashville, TN; Baylor University Medical Center, Dallas, TX; Cook-Fort Worth Children's Medical Center, Fort Worth, TX; Baylor University Medical Center, Houston, TX; M.D. Anderson Cancer Center, Houston, TX; St. Joseph's Hospital Medical Center, Houston, TX; Texas Children's Hospital, Houston, TX; Wilford Hall USAF Medical Center, Lackland, TX; University of Texas Health Science Center, San Antonio, TX; LDS Hospital, Salt Lake City, UT; University of Virginia Medical Center, Charlottesville, VA; Medical College of Virginia, Richmond, VA; Fred Hutchinson Cancer Research Center, Seattle, WA; University of Wisconsin Hospital & Clinics, Madison, WI; Medical College of Wisconsin, Milwaukee, WI.

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Current Status of Bone Marrow Transplantation for Severe Aplastic Anemia: A Preliminary Report from the International Bone Marrow Transplant Registry

E. Gluckman for the Advisory Committee of the International Bone Marrow Transplant Registry

Severe aplastic anemia (SAA) is a usually fatal disease manifested by markedly reduced or absent hematopoiesis. There are numerous causes, but most often the etiology is not known. In most series the etiology can be linked to agents such as drugs, chemicals, virus infections, or total-body exposure to high doses of total-body radiation in 10% to 25% of cases. In other cases, the disease appears to be autoimmune, with the patient's own hematopoietic stem cells as the target. Bone marrow transplantation from an HLA-identical sibling or from a genetically identical twin is generally recognized as a more desirable treatment strategy than alternative therapies. Administration of antithymocyte globulin has also resulted in improvement or cure of the disease, with autologous recovery of hematopoiesis in some cases.

This study was conducted to evaluate the present status of bone marrow transplantation for SAA worldwide. Further, we evaluated

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This is the 39th report from the IBMTR. Address reprint requests to M.M. Borin, MD, Statistical Center, International Bone Marrow Transplant Registry, Medical College of Wisconsin, PO Box 25509, Milwaukee, WI 53226.

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factors that may influence the probability of long-term, disease-free survival with stable engraftment.

PATIENTS AND METHODS

Comprehensive data regarding 455 patients with SAA treated with high-dose chemotherapy with or without radiotherapy and bone marrow transplantation from HLA-identical siblings between January 1, 1978, and June 30, 1985, were reported to the International Bone Marrow Transplant Registry (IBMTR) by 74 transplant teams worldwide. Thirty-four potentially important variables were analyzed to evaluate their effect on the probability of survival with stable engraftment and restoration of hematopoiesis. For this preliminary report we used univariate statistical techniques and standard life table methods to evaluate associations between prognostic and end point variables.

RESULTS AND DISCUSSION

Stable engraftment with partial or full restoration of hematopoiesis following the first transplant occurred in 87% (365/421) of the patients. The actuarial probability of graft failure/rejection at 5 years was 18% ± 5% (95% confidence interval).

Eight factors were associated with the probability of stable engraftment. The incidence of graft failure/rejection was higher ($P < .03$) among patients who received no radiation as part of the conditioning regimen, 17% (35/205) vs 10% (21/216) for patients who were irradiated. Patients treated in conventional isolation had a higher ($P < .05$) incidence (16%, 38/234) of graft failure/rejection than those maintained in laminar air flow isolation (9%, 17/181). A trend ($P < .10$) toward higher graft failure/rejection rates was observed for the following variables: male sex, female donors; multiple fraction vs single fraction radiation; higher pretransplant hemoglobin and platelet counts vs lower readings; longer vs shorter intervals between diagnosis and transplantation; and lower vs higher doses of bone marrow cells per kilogram body weight.

Five factors were identified that were significantly associated with the actuarial probability of 4-year survival following transplantation of bone marrow from HLA-identical siblings for SAA (Table 1). Each of these factors is discussed briefly.

As one might anticipate, a highly significant association exists between engraftment and the probability of survival. On the other hand, although 56 patients failed to achieve stable engraftment following the first transplant, 21 of them are currently alive: 13 had successful second transplants, three had partial restoration of hematopoiesis with improvement of the disease, and five had autologous recovery of hematopoiesis.

Among the patients in whom engraftment is achieved, graft-vs-host disease (GVHD) remains a formidable problem. The 31% (±10%) 5-year actuarial probability of survival for 169 patients who developed moderate-to-severe acute GVHD contrasts with the 80% (±5%) probability of survival among patients with no or only mild acute GVHD. The use of in vitro T cell depletion of donor bone marrow may prove to be an effective means to reduce the risk of GVHD in the future (only one patient in this series received T cell-depleted marrow). Thus far, reports from individual centers have failed to show an improved probability of survival in patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Prognostic Variable</th>
<th>Influence on Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stable engraftment</td>
<td>Unfavorable: No</td>
</tr>
<tr>
<td>2</td>
<td>Acute GVHD</td>
<td>1978-1980 Moderate-severe</td>
</tr>
<tr>
<td>3</td>
<td>Year of transplant</td>
<td>1981-1985</td>
</tr>
<tr>
<td>4</td>
<td>Radiation for conditioning</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>Donor-recipient sex match</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1. Factors Associated With Survival Following HLA-Identical Bone Marrow Transplantation for SAA
receiving T cell-depleted marrow due to an increased incidence of graft failure/rejection. The 62% (±6%) 4-year actuarial probability of survival for 292 patients who received transplants since 1981 vs a 47% (±8%) probability among 163 patients who received transplants earlier suggests that teams and techniques are improving.

Patients receiving radiation as part of their retransplant preparative regimen had a higher \((P < .02)\) probability of survival than those who were not irradiated. This may have been due to the fact that engraftment rates were higher with radiation than without it.

Patients whose donors were of the same sex and a higher \((P < .03)\) probability of survival than when donors and recipients were sex mismatched. The explanation for this finding is uncertain. It is possible that in male-to-male transplants the H-Y antigen on the male cells serves as a target for rejection. In male-to-male transplants, the H-Y antigen may serve as a target for a graft-vs-host reaction. Multivariate analyses are in progress to dress this and other issues.

The analyses reported here provide information regarding the current probability, worldwide, of long-term survival with restoration of hematopoiesis for patients with SAA following treatment with HLA-identical bone marrow transplantation. Several prognostic factors were identified that appear to influence the probability of stable engraftment and disease-free survival.

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Risk factors for interstitial pneumonia following bone marrow transplantation for severe aplastic anaemia

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Summary. Data from 547 patients with aplastic anaemia who received bone marrow transplants from HLA-identical siblings were analysed to determine factors associated with the risk of interstitial pneumonia (IPn). IPn developed in 92 patients (17%). 37% of cases were associated with cytomegalovirus infection and 22% with other organisms; in 41% of cases no organism was identified. The case fatality rate was 64%; the mortality rate due to IPn was 11%. In multivariate analysis, four factors were associated with an increased probability of interstitial pneumonia: use of methotrexate rather than cyclosporine after transplantation (relative risk, 2-8; P < 0-0008); occurrence of moderate to severe acute graft-versus-host disease (relative risk, 2-2; P < 0-002); inclusion of total body radiation in the pretransplant preparative regimen (relative risk 2-2; P < 0-004); and patient age > 20 (relative risk 1-7; P < 0-002). The probability of IPn ranged from 4% for patients with none of these adverse risk factors to 51% (relative risk of 13-4) for patients with all four. The incidence of IPn decreased significantly between 1978 and 1985, paralleling a decrease in the use of total body radiation pretransplant for immune suppression and methotrexate post-transplant for prophylaxis against graft-versus-host disease.

Bone marrow transplantation is an important therapy for severe aplastic anaemia (SAA). Recent data indicate cure rates of 40–70% after bone marrow transplantation from an HLA-identical sibling (Gluckman, 1987; Storb et al., 1980; Anasetti et al., 1986; Storb et al., 1986; Smith et al., 1985; Feig et al., 1983; McGlave et al., 1987). Transplant-related complications associated with failure of bone marrow transplantation in SAA include infection, graft failure and rejection, graft-versus-host disease (GVHD) and interstitial pneumonia (IPn) (Gluckman, 1987; Anasetti et al., 1986; Winston et al., 1984). IPn accounts for up to 40% of treatment failures (Gluckman, 1987; Meyers et al., 1982; Meyers, 1986).

IPn characteristically presents within the first 4 months post-transplant. Typical features include dyspnoea, non-productive cough, progressive hypoxia, bilateral interstitial infiltrates on roentgenograms of the chest and decreased diffusion capacity. Histologic examination of the lung shows oedema, fibrosis, a variable cellular infiltrate, and alveolar exudates (Meyers et al., 1983; Khouria et al., 1979; Sloan et al., 1983). Cytomegalovirus (CMV) is the most common organism associated with IPn: other viruses, Pneumocystis carinii and fungi are less frequent. No organism is identified in 30–60% of patients despite culture and/or histologic examination (Winston et al., 1984; Meyers et al., 1982, 1983; Meyers, 1986).

Previous studies indicate that severe GVHD, older age, and
Pulmonary radiation are important risk factors for interstitial pneumonia following bone marrow transplantation (Meyers et al. 1982, 1983, 1986; Weiner et al. 1986). A recent analysis of 932 patients undergoing allogeneic bone marrow transplantation for leukemia demonstrated that older age, moderate to severe GVHD, use of methotrexate to prevent GVHD, prolonged interval between diagnosis and transplant, poor performance status prior to transplant and, in patients receiving methotrexate as GVHD prophylaxis, higher doses of total body radiation were significant risk factors for IPn (Weiner et al. 1986).

Patients with SAA come to transplantation with medical histories significantly different from patients with acute leukemia. Characteristically, they have received no prior cytotoxic chemotherapy or radiotherapy and have not had many of the consequences of iatrogenic immune suppression. Moreover, the transplant regimen itself differs from that used to treat leukemia since the main thrust is immune suppression rather than antitumour cytotoxicity. It is of interest, therefore, to determine the risk factors for interstitial pneumonia in patients undergoing bone marrow transplantation for SAA.

Most previous studies of IPn after transplantation for SAA included fewer than 100 patients (Gluckman 1987; Storb et al. 1980, 1986; Anasetti et al. 1986; Smith et al. 1985; Peig et al. 1983; McClave et al. 1987). This analysis of 547 patients with SAA who received transplants from histocompatible siblings identified risk factors associated with IPn in this setting. Several significant risk factors were found and a decreasing incidence of IPn between 1978 and 1985 was documented. Hypothesis based on these data and biological principles are suggested to help explain the pathogenesis of this disease.

**Patients and Methods**

Comprehensive data on sequential patients were reported to the International Bone Marrow Transplant Registry (IBMTR) by 84 transplant centres worldwide for 673 patients with SAA transplanted between 1 January 1978 and 31 December 1985. The analysis was based on all data received by 2 December 1986, at which time the minimum follow-up time was 4 months, the maximum 9 years, and the median 2.5 years. Data from the 547 patients receiving bone marrow from HLA A and B identical and mixed leucocyte culture non-reactive sibling donors with no evidence of incompatibility at other HLA loci form the basis for this analysis. Of the 126 patients excluded from analysis, 105 received bone marrow from donors other than HLA identical siblings, 16 received transplants from genetically identical twins, and five were transplanted with tissue other than bone marrow.

Fifty patients received two or more transplants for the following reasons: non-engraftment, 24; incomplete engraftment, six; and late graft failure or rejection, 20. Characteristics of the 547 patients included in the study are presented in Table 1.

All patients were prepared for transplantation using cyclophosphamide. 106 patients also received total body radiation (median dose 3.0 Gy; range 3.0–8.0 Gy) and 165 received limited field radiation (median dose 6.8 Gy; range 1.5–12 Gy). The latter included total lymphoid and thoracoabdominal fields. Donor buffy coat transfusions were given to 148 patients post-transplant. Prophylaxis against GVHD consisted of methotrexate alone or with other drugs in 329, cyclosporine alone or with other drugs in 193, methotrexate in combination with cyclosporine in eight and a variety of other regimens in 17 patients.

In this report, IPn is defined as any nonbacterial pneumonia occurring at any time after transplantation with radiographic and/or histologic features commonly associated with the disease. Biopsy or autopsy findings were available for 51/92 (55%) patients who had IPn.

**Statistical Methods**

A total of 35 prognostic variables were studied (Table II). The association between each risk factor and the occurrence of IPn was tested in univariate analyses using χ² for categorical and Student's t-test for continuous variables. Factors associated with IPn with a P value ≤0.10 in univariate analyses were entered into a multiple logistic regression model in a forward stepwise fashion. Because of the multiple comparisons made, only variables that improved the model with P < 0.01 were considered significant. The probability of IPn for a patient with a single risk factor and with a specific profile of risk factors was derived from the logistic analysis.

The relative risk associated with a single risk factor or profile was computed as the probability of disease for patients with that risk factor or profile divided by the probability of disease for patients with none of the risk factors. All multivariate analyses were examined for possible centre effects in two ways. First, the probability of IPn for patients transplanted in smaller centres was compared with the probability for those transplanted in larger centres. There was no significant difference. Second, transplant centre was entered as a categorical covariate in the multiple logistic regression model. Its inclusion did not significantly improve the model nor did it substantially alter the relative risk or statistical significance of the prognostic variables. All P values are based upon the results of multivariate analysis, unless specified.

**Results**

**Incidence**

Ninety-two of 547 (17%) patients developed IPn. The 3-year
Table II. Peritransplant variables tested for association with the risk of interstitial pneumonia in patients transplanted for severe aplastic anaemia

<table>
<thead>
<tr>
<th>Continuous</th>
<th>Non-continuous</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Patient age</td>
<td>Patient sex</td>
</tr>
<tr>
<td>*Donor age</td>
<td>Donor sex</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>Coexisting disease</td>
</tr>
<tr>
<td>Reticulocyte count</td>
<td>Organ impairment</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>Infected at time of transplant</td>
</tr>
<tr>
<td>Granulocyte count</td>
<td>Trimethoprim-sulphamethoxazole</td>
</tr>
<tr>
<td>Platelets</td>
<td>Intestinal decontamination</td>
</tr>
<tr>
<td>*N transfusions pretransplant</td>
<td>ABO match</td>
</tr>
<tr>
<td>Interval diagnosis to transplant</td>
<td>Sex match</td>
</tr>
<tr>
<td>Performance rating pretransplant</td>
<td>*Isolation procedures pretransplant</td>
</tr>
<tr>
<td>Dose of cyclophosphamide alone</td>
<td>*Radiation field</td>
</tr>
<tr>
<td>Dose of cyclophosphamide + other</td>
<td>Single dose versus fractionated radiation</td>
</tr>
<tr>
<td>Dose of total body radiation</td>
<td>*Drug to prevent graft-versus-host disease</td>
</tr>
<tr>
<td>N fractions of total body radiation</td>
<td>Buffy coat post-transplant</td>
</tr>
<tr>
<td>Dose of limited field radiation</td>
<td>Post-transplant transfusions radiated</td>
</tr>
<tr>
<td>Lung dose of radiation</td>
<td>*Acute graft-versus-host disease</td>
</tr>
<tr>
<td>Dose-rate of radiation</td>
<td></td>
</tr>
<tr>
<td>Unmanipulated cell dose</td>
<td></td>
</tr>
</tbody>
</table>

* Factors associated with interstitial pneumonitis in univariate analysis with \( P < 0.1 \).

Table III. Factors associated with interstitial pneumonitis in multiple logistic analysis

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Favourable</th>
<th>Unfavourable</th>
<th>Relative risk</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug to prevent GVHD</td>
<td>Cyclosporine</td>
<td>Methotrexate</td>
<td>2.8</td>
<td>&lt;0.0008</td>
</tr>
<tr>
<td>Radiation for pretransplant conditioning</td>
<td>None or limited field</td>
<td>Total body</td>
<td>2.2</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>None or mild</td>
<td>Moderate to severe</td>
<td>2.2</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Patient age (years)</td>
<td>( \leq 20 )</td>
<td>&gt;20</td>
<td>1.7</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

actuarial probability of IPn was 21 ± 4% (95% confidence interval). The interval between transplantation and onset of IPn was 79 ± 65 d (mean ± SD). 80% of cases occurred within the first 120 d after transplantation. Thirty-four (37%) of 92 cases were associated with CMV and 20 (22%) with other organisms (Pneumocystis carinii in six patients, Aspergillus sp. in six patients, and various other organisms in eight patients). No organism was identified in 38 (41%) patients.

Risk factors

Four of the seven variables associated with development of IPn in univariate analysis (Table II), were shown to be significant adverse risk factors in multivariate analysis: use of methotrexate as prophylaxis against GVHD, development of moderate to severe acute GVHD, use of total body radiation for conditioning, and older patient age (Table III).

Methotrexate was strongly associated with the development of IPn (Fig 1A, \( P < 0.0008 \)). The risk of interstitial pneumonia was 2-8 times greater for patients who received methotrexate with or without other drugs than for those who received cyclosporine with or without other drugs to prevent GVHD. The number of patients who received other regimens to prevent GVHD was too small to assess their impact upon the probability of IPn.

The effect of radiation on the incidence of IPn is shown in Fig 1B. Conditioning regimens that included total body irradiation were associated with a risk of IPn 2.2 times greater than conditioning regimens using either limited field radiation (total nodal, total lymphoid or thoracoabdominal irradiation) or no irradiation (\( P < 0.004 \)). While conditioning regimens that included limited field radiation were associated with a higher risk of IPn (relative risk 1.5) than those using cyclophosphamide alone, this difference was not statistically significant (\( P > 0.1 \)). For all further analyses, therefore, the patients receiving limited field irradiation were combined with those receiving no irradiation.

The patients receiving total body radiation did not differ significantly in age or incidence of acute GVHD from patients who were conditioned without total body radiation. Because both methotrexate and total body radiation were associated with an increased risk of IPn, we examined this interrelation-
ship in greater detail. Methotrexate was used in 79% (84/106) of patients receiving total body radiation as compared to 56% (245/441) of those who received limited field or no radiation (univariate P<0.001). In order to test the association between methotrexate and IPN in the absence of pulmonary irradiation, the 441 patients who received limited field or no irradiation were analysed as a separate subset by multiple logistic regression analysis. The 245 patients who received methotrexate had a relative risk of 2.5 compared to the 196 patients who received no methotrexate (P<0.004).
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Moderate to severe acute GVHD was also associated with the risk of IPn (Fig 1D, P < 0.002). Fifty-five of the 92 patients with IPn had moderate to severe acute GVHD: manifestations of GVHD were documented to have preceded the onset of IPn in 51 (93%) of these 55 cases. Individuals with moderate to severe acute GVHD had a relative risk of IPn of 2.2 compared to patients with no or mild acute GVHD. The incidence of acute GVHD was not significantly different in patients who received methotrexate as opposed to cyclosporine for GVHD prophylaxis (37% v. 42%, P = 0.35).

Older patient age was a significant risk factor for IPn; age > 20 years was associated with relative risk of 1.7 (Fig 1E, P < 0.002). The incidence of moderate to severe acute GVHD was not significantly different between patients ≤ 20 and > 20 (36 v. 42%, P = 0.13).

Fig 2 illustrates the cumulative effect of these variables on the probability of IPn. The probability of IPn was 51% for the group of patients who received methotrexate, developed moderate to severe acute GVHD, received total body irradiation for immune suppression pre-transplant and were older than 20 years of age at the time of transplant. Among patients with none of these risk factors, the probability of IPn was only 4%. The total number of cases of IPn was too small to allow meaningful logistic analysis of risk factors for idiopathic IPn.

Case fatality and mortality rates
In 59 of the 92 patients with IPn, the complication was either a primary or contributory cause of death resulting in a case-fatality rate of 54%. The case fatality rate was 47% (18/38) for patients with idiopathic IPn, 74% (25/34) for patients with CMV IPn and 80% (16/20) for patients with IPn associated with other organisms. Overall, IPn accounted for 26% of the 228 fatalities in this study. IPn was the primary or a contributory cause of death in 11% of the patients (59/547).

IPn developing in the setting of GVHD was more frequently fatal. While 75% of patients with both IPn and moderate to

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Table IV. Change in per cent incidence of interstitial pneumonitis and related factors with time

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Incidence of interstitial pneumonitis (%)</td>
<td>26</td>
<td>20</td>
<td>11</td>
<td>14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Methotrexate used (%)</td>
<td>90</td>
<td>82</td>
<td>47</td>
<td>32</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Radiation field used (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>52</td>
<td>46</td>
<td>54</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Limited field</td>
<td>16</td>
<td>31</td>
<td>32</td>
<td>43</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Total body</td>
<td>33</td>
<td>23</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Moderate to severe acute GVHD (%)</td>
<td>51</td>
<td>36</td>
<td>44</td>
<td>38</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Median patient age (years)</td>
<td>18</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Organisms identified (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>43</td>
<td>49</td>
<td>43</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>48</td>
<td>35</td>
<td>29</td>
<td>33</td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>15</td>
<td>28</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Case-fatality rate (%)</td>
<td>61</td>
<td>67</td>
<td>71</td>
<td>53</td>
<td>&gt;0.1</td>
</tr>
</tbody>
</table>
severe acute GVHD died with IPn as a primary or contributory cause of death. 51% with IPn and either no or mild acute GVHD died of IPn (univariate $P < 0.03$).

Decreasing incidence

The overall incidence of IPn decreased over the 8 years of this study. The incidence was 22% during the years 1978–81 and 12% from 1982 to 1985 (univariate $P < 0.01$). This decrease paralleled the significant decrease in the use of methotrexate for prophylaxis of GVHD and the significant decrease in the use of total body radiation for conditioning (Table IV). The association between decreased incidence of IPn with temporal changes in treatment is further strengthened by the fact that there was no change in the incidence of IPn during this period for patients who received both pulmonary irradiation and methotrexate nor was there a change in incidence of IPn for those patients who received neither pulmonary irradiation nor methotrexate. The incidence of moderate to severe GVHD, the age of transplanted patients, the spectrum of organisms identified and the case-fatality rate of IPn did not change significantly between 1978 and 1985.

DISCUSSION

This analysis of 547 patients transplanted for SAA demonstrates several important risk factors for the development of IPn. These include two major treatment variables: the use of total body irradiation in the pre-transplant conditioning regimen and the use of methotrexate for GVHD prophylaxis. Decrease in the use of total body radiation and of methotrexate were associated with a decreasing incidence of IPn during the 8-year study period.

Incidence and fatality of IPn

We and others reported previously that the incidence of IPn in patients transplanted for SAA is lower than among patients transplanted for leukemia (Meyers et al. 1982, 1986; Weiner, 1987). The actuarial probability of IPn was 21% in this study, significantly lower (univariate $P < 0.0001$) than the 35% probability among 932 patients with leukemia reported to the IBMTR by essentially the same transplant teams during a similar time period (Weiner et al. 1986). The case fatality rate of IPn was also lower in SAA than in leukemia (64% v. 84%, univariate $P < 0.0001$). This difference was due primarily to the lower case-fatality associated with idiopathic IPn in SAA patients: 47% v. 78%. CMV IPn was usually lethal in both SAA and leukemia (Weiner et al. 1986; Weiner, 1987). Both the higher incidence and higher case-fatality rates in leukemia patients might be explained by the chemotherapy given to leukemia patients during the treatment of their disease. Many of these drugs cause pulmonary toxicity and prolonged immune suppression (Ginsberg & Comis, 1982; Whitcomb, 1983; Haupt et al. 1981). Conditioning regimens for leukemia patients often use higher doses of radiation and drugs with antileukemia activity. As a consequence, conditioning regimens used prior to transplantation in leukemia may be more toxic to the lung than preparative regimens used in SAA (Haupt et al. 1981; Barratt et al. 1983; Pino et al. 1982).

Drugs to prevent GVHD

Most recipients of allogeneic bone marrow transplants receive prophylaxis against GVHD. Methotrexate or cyclosporine regimens are the most widely used. This study identified a highly significant association between the use of methotrexate-containing regimens and IPn. A similar association was found in our analysis of IPn in leukemia patients (Weiner et al. 1986). In the current study, this association was significant even in the subgroup of patients who did not receive total body radiation (and thus did not have pulmonary radiation), suggesting a direct toxic effect of methotrexate on the lung or perhaps one mediated through an interaction with cyclophosphamide (Chan et al. 1979; Demeter et al. 1979; Sostman et al. 1976). Two randomized studies comparing methotrexate with cyclosporine as prophylaxis against GVHD in patients receiving pulmonary radiation did not identify an increased risk of IPn in those patients given methotrexate (Deeg et al. 1985; Forman et al. 1987). This disparity may be explained by the inherent difficulty in identifying significant risk factors (high beta error) in small series of patients.

Effect of radiation

A number of studies have suggested that pulmonary radiation plays a role in the pathogenesis of IPn (Barrett et al. 1983; Pino et al. 1982; Chan et al. 1979; Keane et al. 1981; Peters et al. 1979; Bortin & Harts. 1988). We found no relationship in leukemia patients (98% of whom received total body radiation) between radiation dose and IPn with lung doses between 5.6 and 12.8 Gy (Weiner et al. 1986). However, dose-rates of radiation in excess of 5 cGy/min were associated with a significantly increased risk of IPn only if methotrexate was given as prophylaxis against GVHD. In contrast to leukemia, preparative regimens for bone marrow transplantation in SAA often do not include pulmonary radiation (Anasetti et al. 1986; McGave et al. 1987). Therefore, we were able to evaluate the effect of radiation on the risk of IPn.

The field of radiation made a significant difference regarding the risk of IPn in patients with SAA. Those receiving total body irradiation, i.e. a radiation field including the lungs, had a significantly increased risk of IPn. A dose response effect of total body irradiation; however, was not identified. The range of doses was relatively small with 75% of patients receiving between 3 and 4.5 Gy. Moreover, there was no effect on dose-rate of irradiation. It may be that dose-rate is not as important when the total dose is low. Eliminating pulmonary radiation from the transplant regimen would, it appears, reduce significantly the risk of IPn.

Interaction between radiation and drugs to prevent graft-versus-host disease

The patients receiving cyclosporine for prevention of acute GVHD did not demonstrate a significantly increased incidence of IPn when total body irradiation was part of the transplant regimen (Fig 1C). However, the number of patients receiving cyclosporine without total body irradiation was too small to exclude the possibility that an effect of total body irradiation might be present but not apparent. The use
of total body irradiation in patients receiving methotrexate, however, was associated with a significantly increased incidence of IPn in comparison with the use of methotrexate without radiation (Fig 1C). These data indicate that methotrexate is associated with a markedly increased risk of IPn and that this risk is even greater when methotrexate is used in patients who received total body irradiation. These observations are in accord with others that methotrexate and radiation act synergistically to increase pulmonary toxicity (Chan et al. 1979; Phillips & Fu, 1978).

The data support the hypothesis that injury to the lungs is an important pathogenic mechanism of IPn and that avoiding pulmonary toxins reduces the risk of IPn after allogeneic bone marrow transplantation. These findings suggest that the risk of interstitial pneumonia in severe aplastic anaemia patients undergoing transplantation would be reduced if total body irradiation were excluded from the conditioning regimen, especially for those patients who are to receive methotrexate as prophylaxis for acute graft-versus-host disease. Moreover, the risk of interstitial pneumonia could be reduced even for those patients not receiving pulmonary radiation if methotrexate were not used as prophylaxis for acute graft-versus-host disease. It must be borne in mind, however, that preparative regimens without radiation may be associated with an increased risk of graft failure in severe aplastic anaemia (Champlin et al. 1988). Further study is required before recommendations can be made with respect to the use of radiation for pretransplant conditioning of patients with severe aplastic anaemia.

**Effect of acute GVHD**

Acute GVHD is associated with an increased risk of IPn (Meyers et al. 1986; Weiner et al. 1986). This study also demonstrates that the risk of IPn is significantly higher in patients who develop moderate to severe acute GVHD. Several factors may play a role in this association. First, GVHD itself causes profound immune suppression and is often treated with drugs that may further decrease immune responsiveness (Ellenbein, 1983). Thus, GVHD may impair host defences against endogenous or exogenous CMV, as well as other microorganisms. Second, GVHD may activate latent CMV re-infection (Meyers et al. 1986). In addition to being more common, IPn was more likely to be fatal in patients with coincident moderate to severe acute GVHD. Only 25% of individuals had resolution of IPn in this setting compared to 51% of patients with nor or mild acute GVHD.

**The effect of age**

Increasing age had a significant adverse effect upon the risk of developing IPn. This age effect most likely reflects the increased probability of prior CMV infection in older patients, and, therefore, the increased possibility that endogenous CMV will be activated post-transplant (Meyers et al. 1986: Ho. 1982). Data on CMV antibody status pre-transplant were not available for patients in this study precluding an analysis based on positive CMV serology. Correlations between age, CMV seropositivity, and the risk of CMV IPn have been reported (Meyers et al. 1986).

The effect of age on risk of IPn does not appear to be mediated through GVHD. When the relative risk associated with age > 20 derived from the multiple logistic equation was adjusted for the effect of acute GVHD, the two covariates were not significantly correlated.

**Differences between leukaemia and SAA patients**

Many of the findings of this study parallel those in 932 leukaemia patients reported to the IBMTR during the same time period (Weiner et al. 1986). Methotrexate, acute GVHD and older age were significant risk factors for IPn in both populations. The role of radiation in increasing the risk of IPn suggested in the previous study, was more clearly demonstrated in the current study since a large number of patients who received no pulmonary radiation were available for comparison. The present results demonstrate that age, methotrexate and GVHD remained significant risk factors even in the absence of pulmonary radiation.

Two variables which were significant risk factors for IPn in leukaemia were not significant in this analysis: Karnofsky performance rating pretransplant and the interval from diagnosis to transplant. Since both of these variables are affected by the duration and intensity of pretransplant antineoplastic treatment, their significance in leukaemia patients may reflect cumulative pulmonary toxicity and/or prolonged immune suppression.

**Trends in the incidence of IPn and practice variables**

The incidence of IPn declined significantly between 1978 and 1985. Two changes in clinical practice which paralleled this decrease were identified. More than twice as many patients received methotrexate or total body irradiation in 1978–79 as in 1984–85. Other factors that might account for the decreasing incidence of IPn over the past 8 years were tested but none were identified. Further decreases in this important cause of morbidity and mortality following bone marrow transplantation for SAA may be possible on the basis of the findings reported here.

**ACKNOWLEDGMENTS**

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Pneumonia after BMT for Aplastic Anaemia


Qualitative Abnormalities Characterize Hematopoiesis that Restores Marrow Function after Therapy with Antilymphocyte Globulin and High-Dose Methylprednisolone in Aplastic Anemia

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Purpose: Antilymphocyte globulin (ALG) can restore bone marrow function in patients with aplastic anemia, but following recovery, hematopoiesis may be functionally abnormal. The extent of these defects was retrospectively characterized in a uniformly treated cohort of patients that had been followed up for between 1 and 8 years.

Patients and Methods: Thirty-four patients with a median age of 25.5 years (SD 15.66) received a standard regimen of anti-human lymphocyte globulin together with high-dose methylprednisolone on 5 consecutive days, followed by oxymetholone for 6 to 9 months. The initial response rate was 71%, but conditions of 6 patients deteriorated, 2 patients required a second course of ALG, 2 patients are cyclosporin-dependent and 1 patient continues to receive androgenic therapy. Standard techniques were used to assess peripheral blood and bone marrow morphology and bone marrow cytogenetic status. In 16 responders, function in the granulocytes was defined by phagocytosis, intracellular killing and superoxide generation. Lymphocytes were characterized by response to mitogens and alloantigen and immunoglobulin production and platelets by bleeding time and aggregometry.

Results: At a median of 4 years mild granulocytopenia and thrombocytopenia were detected, with dysplasia present in all but three patients. Two developed paroxysmal nocturnal hemoglobinuria. Chromosomal breaks and gaps were demonstrable in one-third of patients in 13 to 40% of metaphases studied. Neutrophil functions were normal in only one patient, while lymphocyte studies were adequate in all but two who were receiving cyclosporin therapy; bleeding times were abnormal in two-thirds of the patients while aggregometry was universally defective.

Conclusions: The combination of ALG and high-dose methylprednisolone produced variable hematologic responses in 71% of patients and although two-thirds of these are currently alive with only mild quantitative defects, considerable qualitative abnormalities are present in the majority. These findings reflect the persistence of a substantially abnormal hematopoiesis.

INTRODUCTION

Irreversible bone marrow failure[1] is usually idiopathic but may be associated with exposure to drugs,[2] pregnancy,[3] jaundice[4,5] and infections. The variable clinical expression reflects the extent of the hematopoietic damage and although mild or moderate hypoplasia may respond to andro-
genic steroids, these are ineffective in severe disease. Here patients will benefit from allogenic bone marrow transplantation, particularly young and unsensitized individuals.

Where this option is not available and based on observations that immune mechanisms may suppress hematopoiesis, antilymphocyte globulin (ALG) in combination with corticosteroids, cyclosporin and anabolic androgens elicits responses in more than 50% of patients. However, relapses occur even in the face of normal peripheral blood values. Furthermore, dysplasia, defective platelet function, alterations in T-lymphocyte subsets and reduction in the serum immunoglobulins have also been reported.

These morphologic features may be similar to those described in patients with myelodysplastic syndromes (MDS), who have hypoplastic bone marrow and who also respond to immunosuppressive therapy. This confusion is further compounded when patients with typical aplasia develop MDS, acute leukemia or paroxysmal nocturnal hemoglobinuria (PNH). To explore this interrelationship, we have studied a series of patients with aplastic marrow, who received uniform immunosuppressive therapy, and documented the morphologic, cytogenetic and functional characteristics of their hematopoiesis over 8 years of follow-up.

PATIENTS AND METHODS

From January 1985 to December 1991, 34 individuals with severe aplastic anemia and one with hypoplasia, 15 of whom had been previously reported, were referred for therapy. None had phenotypic features of Fanconi’s anemia; presentation had been during late adolescence and adulthood and without exception, the disease had been of a rapid onset. The median age was 25.5 years (range 13 to 72; SD 15.66) and 17 were female. Two had convalescent serology for hepatitis B; one was pregnant but recovered partially after a therapeutic abortion. One had been exposed to chloramphenicol in eye drops and another received chloroquine for rheumatoid arthritis, and neither recovered after drug discontinuation.

At presentation, the median value for hemoglobin (Hb) was 66 g/l (SD 20.9), the corrected reticulocyte count was 0.3% (SD 0.29), the mean red cell volume was 102 fl (SD 54.76), the granulocyte count was $0.50 \times 10^3/\text{l}$ (SD 0.44) and the platelet count was $11.5 \times 10^3/\text{l}$ (SD 5.37). Median bone marrow cellularity was 15% (SD 5.4) of normal. Vitamin B$_{12}$ and red cell folate status was normal.

Therapy

After confirmation of the diagnosis, individuals were entered into a standard protocol approved by the University of Cape Town and Groote Schuur Hospital Ethics and Research Committees, consisting of ALG (horse immunoglobulin without thrombocyte adsorption, Swiss Serum Institute, Bern, Switzerland) 50 mg/kg, and high-dose methylprednisolone, 500 mg daily on each of 5 consecutive days, followed by 30 mg of prednisone daily for 1 month. Response was defined as reversal of symptoms, freedom from transfusions, a stable rise in the platelet count above $40 \times 10^3/\text{l}$ and an absolute granulocyte count $1 \times 10^3/\text{l}$. Complete remission required Hb $>$120 g/l, granulocytes $>$2.5 $\times 10^9/\text{l}$ and platelet count $>$150 $\times 10^9/\text{l}$. Patients with inadequate responses or with fluctuating blood counts received oxymetholone 2 mg/kg for 6 to 9 months.

Laboratory Investigations

The current analysis was performed on a cohort of patients with severe aplasia who had responded to immunosuppressive therapy and who were investigated at a median of 4 years (range 1 to 8) from initial presentation.

Peripheral Blood and Bone Marrow Examination. Routine hematological studies were done
by standard methods. Bone marrow aspirates were stained with Romanowsky dyes and reviewed retrospectively, with special search for dysplastic features according to the French-American-British criteria. Trephine biopsies were sectioned and stained with hematoxylin and eosin and evaluated for inflammatory infiltrates, abnormalities in the stroma or abnormal localization of immature precursors (ALIP).

Cytogenetic Analysis. Bone marrow chromosome analysis was performed on trypsin (Difco, Detroit, MI) Giemsa banded metaphases. When necessary, phytohemagglutinin (PHA) (Murex Diagnostics Ltd, England)-stimulated blood cultures were carried out to establish the constitutional cell line.

Granulocyte Functions. Peripheral blood leukocytes were obtained from 13 patients with aplastic anemia and 9 concurrent control subjects by dextran sedimentation (dextran 6% w/v in 0.9% NaCl) of heparinized blood (20 U/ml) and divided in aliquots for the various assays. Determination of superoxide generation, and the combined measurement for phagocytosis and killing were performed following previously described methods. Chemotaxis was studied according to Addison and Babbage.

Lymphocyte Functions and Serum Immunoglobulin Levels. Peripheral blood mononuclear cells from patients with aplasia and control subjects were washed twice in RPMI-1640 (Gibco) and 0.1 ml of the suspension (5 x 10⁶/ml) was cultured for 3 days at 37°C in 96-well microtiter plates (Cel-Cult, Sterilin Ltd., Hounslow, UK) in the presence of 0.05 ml of concanavalin A (Calbiochem, La Jolla CA), PHA, pokeweed mitogen (Gibco) or heat-inactivated AB serum (WPBTS). Cells were then pulsed with 1 mCi of [³H]thymidine (Amersham, 1:200) at 37°C and harvested 18 hours later on to dry filter discs, and the radioactivity was counted. For the mixed lymphocyte reaction, allogeneic control lymphocytes were thawed and incubated at 37°C for 20 min in mitomycin (MO 0503, 1:8 dilution, Sigma, St. Louis, MO). After being washed twice in medium, 0.1 ml of the cell suspension (5 x 10⁶/ml) was cultured in triplicate with patient or control lymphocytes for 5 days and pulsed with 1 mCi of [³H]thymidine (Amersham) for 18 hr. Cells were then harvested onto filter discs, and radioactivity was measured on a beta scintillation counter (Beckman).

Immunoglobulin levels were evaluated by agar electrophoresis. Quantification of IgG, IgA and IgM was performed by single radial immunodiffusion using immunodiffusion plates, and the results were compared to a normal range.

Bleeding Time and Platelet Aggregometry. Platelet functional activity was determined only for patients with platelet counts > 100 x 10⁴/l and compared with control values. Both groups were instructed to avoid medications that could interfere with platelet function for 2 weeks preceding the study. Bleeding time was determined by the Ivy method and at the same time blood was collected for aggregometry into siliconized vacutainer tubes containing sodium citrate. Comparisons were carried out in platelet-rich plasma, using a Chronolog aggregometer (Chronolog Corp., Havertown PA) in the presence of agonists [ADP, 2 mg/ml of collagen or 1.5 mg/ml of ristocetin (Sigma) and epinephrine (May Baker, South Africa)], and the results were registered on a chart recorder (models 560vs and 707, Chronolog).

Statistical Analysis
Patient survival was measured by the Kaplan-Meier method. Median values and confidence intervals (confidence coefficient: 0.95) of the patient results were calculated and compared with control results. The significance was tested by two-way analysis of median values.
Response to Therapy

In the patient with hypoplasia, remission occurred after a therapeutic abortion, and as she was not treated, she was excluded from analysis of response to therapy. Responses were seen in 24 of the 34 subjects (71%), and were complete in 8. At a median follow up of 1320 days (SD 803), 22 subjects (65%) are alive in response (Fig. 1). Although hematological responses were seen at a median of 110 days (range 60 to 300), improvement in the blood counts continued for up to 4 years after therapy. Four patients received oxymetholone within a month of ALG infusion for a median of 6 months. However, another 9 required this androgenic steroid for a median period of 6 (range 1 to 18) months due to fluctuations in the blood values. In 4 patients, this hormonal therapy alone resulted in blood count improvements. Six of 24 patients (25%) had relapses; but 4 are alive. In one, disease improved following the delivery of a normal infant. Three other patients received oxymetholone and further immunosuppression and are in their second (1 patient) or third response (2 patients, with 1 being sustained on cyclosporin maintenance therapy).

None of the clinical findings at presentation predicted the response. Among the pretreatment laboratory data, only a mean corpuscular volume >95 fl was significantly associated with response (responders, median 109 fl, SD 10.1 versus nonresponders 91 fl, SD 7.6; median test p = 0.015, exact two-tailed).[12,28]

RESULTS

Morphological Assessment

Of 22 patients alive in response after immunosuppressive therapy and one after therapeutic abortion, at a median of 4 years from presentation (range 1 to 8), 16 were available and agreed to undergo the present study. At the time of this study, 2 patients were receiving cyclosporin and 1 oxymetholone therapies due to recurrence of pancytopenia. Review of the blood and marrow

![SURVIVAL OF PATIENTS WITH APLASTIC ANAEMIA](image)

FIGURE 1 Survival of 34 patients with aplastic anaemia after ALG + high-dose methylprednisolone. Thirteen patients received androgens for suboptimal response or falling blood values (see "Results"). Twenty-two patients remain in response at a median of 4 years follow-up.
morphologic findings from the initial admission of these 16 individuals revealed mild megaloblastosis limited to the red cell precursors in 13 patients, while in another, in addition, slight abnormalities in the granulocytic series with giant bands, were observed. Cytologic findings were entirely normal in 1 patient and in 2 patients there was no analyzable hematopoietic tissue.

Reassessment of this group of 16 subjects performed at a median of 4 years from therapy showed continued improvement of the blood values. However, considerable morphologic abnormalities were also detected, characterized by dyserythropoiesis, megaloblastosis, giant band cells and Pelger-Huet forms (Tables I and II). In the bone marrow no excess of blasts or inflammatory infiltrates or increase in mast cells was detected. Iron stores were absent in 1 patient while pathologic or ringed sideroblasts were not seen in any. Median cellularity was 40% on biopsy. Morphologically, trilineage dysplasia with ALIP was present in 3 patients (Table II), and in 2 of them, a patchy increase in reticulin fibrosis was also observed. Two patients developed a positive Ham’s test with clinical features of PNH.

Cytogenetics

Metaphases suitable for analysis were obtained in 15 of 16 individuals. One patient had an abnormal

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<thead>
<tr>
<th>Peripheral blood</th>
<th>Observation period</th>
<th>Median</th>
<th>SD</th>
<th>Median</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 yr</td>
<td>4 yr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>115.00</td>
<td>124.50</td>
<td>27.21</td>
<td>25.30</td>
<td>22.1</td>
</tr>
<tr>
<td>Granulocytes (x 10^9/l)</td>
<td>1.70</td>
<td>2.21</td>
<td>1.89</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x 10^9/l)</td>
<td>0.92</td>
<td>1.53</td>
<td>0.72</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Monocytes (x 10^9/l)</td>
<td>0.15</td>
<td>0.23</td>
<td>0.18</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>88.00</td>
<td>127.00</td>
<td>80.44</td>
<td>80.00</td>
<td>80.00</td>
</tr>
</tbody>
</table>

TABLE I  Quantitative Evaluation of the Blood Parameters of Patients with Aplastic Anemia at a Median of 1 and 4 Years after Immunosuppressive Therapy

karyotype expressed as 47,XXX in 8 of 12 bone marrow metaphases, the remainder showing random chromosomal loss. PHA-stimulated peripheral blood analysis confirmed the constitutional nature of the trisomy X. Chromosomal breaks and/or gaps were present in another 5 of 15 patients, ranging from 13 to 40% of cells tested. No other cytogenetic abnormality was detected. In one patient in whom karyotypic analysis of lymphocytes was performed, it did not show such chromosomal breaks. However, the studies were not executed in the presence of clastogenic agents to exclude the diagnosis of Fanconi’s anaemia. At the time of the current study, none were receiving any therapy. The clinical data for these 5 patients are presented in Table III.

Granulocyte Functions

Only 1 of the 13 patients studied had normal granulocyte functions, while in 3, all assays were deranged. In the remaining subjects, results were below control values in at least one assay. As a
group, median values were significantly reduced for superoxide generation ($p = 0.007$) and phagocytosis ($p = 0.002$) (Table IV). No correlation was found between blood leukocyte counts and \textit{in vitro} granulocyte functions or any other laboratory or clinical data, including age, sex or time from therapy.

**Lymphocyte Functions and Immunoglobulins**

All functional measurements were normal in 12 patients, but subnormal in both patients receiving cyclosporin therapy. Serum immunoglobulins were determined in 14 patients, and values were found within the standard range in all, including both patients receiving maintenance immunosuppression.

**Platelet Functions**

The platelet count was $>100 \times 10^9/l$ (median 127, SD 80.44 $\times 10^9/l$) in 14 patients. However, the Ivy time was normal in only 5 of these individuals. For all patients the median bleeding time was 8.5 min (SD 3.4) (normal range 3 to 7 minutes) and in 3 it was $>15$ min. Aggregation studies were significantly abnormal, and this applied to each of the agonists tested (Table V).

**TABLE III. Clinical Characteristics of 5 Patients with Aplasia and Cytogenetic Abnormalities after Immunosuppressive Therapy**

<table>
<thead>
<tr>
<th>Age at presentation (yr) and sex</th>
<th>Time from ALC to investigations</th>
<th>Immunosuppression</th>
<th>Hb (g/l)</th>
<th>White cells ($\times 10^9/l$)</th>
<th>Platelets ($\times 10^9/l$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16, M</td>
<td>1 yr</td>
<td>ALC; Oxy for 4 mo</td>
<td>14.7</td>
<td>4.2</td>
<td>169</td>
</tr>
<tr>
<td>20, M</td>
<td>4 yr</td>
<td>ALC; Oxy for 11 mo</td>
<td>15.3</td>
<td>2.9</td>
<td>146</td>
</tr>
<tr>
<td>33, F</td>
<td>6 mo</td>
<td>Oxy for 4 mo</td>
<td>13.5</td>
<td>3.7</td>
<td>111</td>
</tr>
<tr>
<td>47, F</td>
<td>6 yr</td>
<td>ALC; Oxy for 18 mo</td>
<td>9</td>
<td>3.8</td>
<td>50</td>
</tr>
<tr>
<td>55, F</td>
<td>5 yr</td>
<td>ALC; Oxy for 3 mo</td>
<td>13.7</td>
<td>3.1</td>
<td>119</td>
</tr>
</tbody>
</table>

*All patients received oxymetholone (Oxy) at some stage, but none were receiving this therapy at the time of the investigations.

**TABLE IV. Granulocyte Functions of Patients with Aplastic Anemia Tested at a Median of 4 Years after ALC Therapy and Compared with Concurrent Control Subjects**

<table>
<thead>
<tr>
<th>Functions tested</th>
<th>Patients (n = 12)</th>
<th>Control subjects (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>SD</td>
</tr>
<tr>
<td>Generation of peroxide (mmol/min/10^9 cells)</td>
<td>2.79*</td>
<td>0.60</td>
</tr>
<tr>
<td>Chemotaxis (mm)</td>
<td>83.00</td>
<td>33.97</td>
</tr>
<tr>
<td>Migration (mm)</td>
<td>65.00</td>
<td>17.38</td>
</tr>
<tr>
<td>Phagocytosis (%)</td>
<td>53.10*</td>
<td>12.89</td>
</tr>
<tr>
<td>Killing (%)</td>
<td>32.00</td>
<td>18.61</td>
</tr>
</tbody>
</table>

*Significant at $p < 0.05$.
**NS, not significant.
TABLE V  Platelet Aggregation Studies Performed on Patients with Aplastic Anemia at a Median of 4 Years after ALG Therapy

<table>
<thead>
<tr>
<th>Aggregation studies</th>
<th>Patients (n = 14)</th>
<th>Control subjects (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>SD</td>
</tr>
<tr>
<td>ADP 100</td>
<td>56.03*</td>
<td>19.94</td>
</tr>
<tr>
<td>ADP 10</td>
<td>47.50*</td>
<td>20.07</td>
</tr>
<tr>
<td>ADP 5</td>
<td>31.00*</td>
<td>15.98</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>10.00</td>
<td>20.13</td>
</tr>
<tr>
<td>Collagen</td>
<td>35.00*</td>
<td>31.70</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>69.50*</td>
<td>19.28</td>
</tr>
</tbody>
</table>

*Median platelet count: 127 x 10⁹/L; SD 89,44.  
*Significant at p < 0.05.

DISCUSSION

This study confirms previous observations that ALG is effective in improving the pancytopenia associated with idiopathic bone marrow failure.⁷¹,² The number of variables including patient age,³⁵ differences in disease severity,²³ or the intensity of the immune suppression³¹-³³ may also explain the unpredictable outcomes reported with this modality. Macrocytosis was the only feature significantly associated with a favorable response.¹²,²⁸ However, mild megaloblastic changes progressed to bilineage (8 patients) and trilineage (3 patients) dysplasia and increased deposition of reticulin and even ALIP over the follow-up period. The significance of these observations remains unclear, although prominent dysmyelopoiesis has been associated with an increased risk of progression to leukemia.¹⁹ Whether within this group, patients with morphologic but not quantitative abnormalities should also be regarded as having myelodysplasia remains questionable.

Furthermore, karyotypic derangements may provide a clue to the possible link between aplasia and myelodysplasia,³⁴,³⁶ and the present demonstration that 13 to 40% of metaphases in 5 of 15 individuals were abnormal, supports the concept that disturbed DNA repair mechanisms may presage evolution to the preleukemic syndrome.¹⁸ Nevertheless, the natural history of MDS that follows aplasia may be different from the de novo variant, with reduced or delayed evolution to more advanced types or into acute leukemia.³⁷ Moreover, childhood MDS, although an infrequent malignancy, may present with hypoplastic marrow in a proportion that is not different from the adult variant³⁸-⁴¹ and may also respond to immunomodulation, thus further adding to the confusion.⁴² Similar to the adult experience, in responsive patients with aplasia, dysmyelopoiesis³⁹,⁴³ and progression into clonal disorders, have also been reported to occur in the pediatric population,⁴⁰ suggesting that analogous pathogenetic mechanisms may be operating. This is particularly relevant to this age group because of the extended period of life expectancy and therefore increased risk of clonal progression. Lastly, in the present group the diagnosis of Fanconi's anemia was felt to be unlikely, owing to the lack of somatic defects, the age of presentation and the acute onset of the disease. However, no patient was subjected to diepoxybutane testing.²⁵

Defective granulocyte function was reflected in significantly reduced superoxide generation and phagocytosis, although it was not associated with increased infective episodes. This may perhaps represent normal lymphocyte function and immunoglobulin levels resulting from a more complete immunologic reconstitution. We have also confirmed that despite continuous improvement in the cellularity during sequential testing, mild thrombocytopenia persisted in the majority. As in this disease platelet survivals were shown
to be normal, the reduction in the thrombocyte numbers possibly reflected decreased production or ineffective thrombopoiesis.\textsuperscript{14} However, despite platelet counts exceeding $100 \times 10^9$/l in 14 patients, a value not associated with purpura, the bleeding time was prolonged in 7. Not surprisingly, significant aggregation abnormalities were detected.

With the pathogenesis of idiopathic marrow aplasia remaining obscure, our findings are consistent with two broad possibilities. First, the initial insult may have irreversibly damaged hematopoietic stem cells, and this was reflected as qualitative defective mature progeny. Alternatively, the differentiating population was subjected to continuous aggression by subclinical immunologically mediated mechanisms\textsuperscript{15-18} in the hematopoietic microenvironment.\textsuperscript{19-21} The former hypothesis would appear to be more likely since we have demonstrated that the aplastic stroma supports the growth of normal selected progenitors adequately and that the defect resides predominately within the aplastic precursors.\textsuperscript{22,23}

It is concluded that while response may follow immunosuppressive therapy for severe marrow aplasia, this quantitative recovery is coupled with chromosomal abnormalities and trilineage morphologic and functional alterations. In some patients this reflects the persistence of an unstable progenitor cell population with considerable risk of evolution into any of the described clonal disorders.

Acknowledgments

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We thank Jackie Davis for typing and Jessica Gerretsen for comprehensive editorial assistance.

References

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Acquired Aplasia of the Bone Marrow

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Supported by the University of Cape Town Leukaemia Centre and Staff Research (Footie, Becker and Cancer) Fund, the Gwendoline Moore Trust, the Cancer Association of South Africa, the Medical Research Council and the Michael Chanani, Kalinski and M A Richardson Bequests.

Keywords: Aplastic Anaemia; Pathogenesis; Treatment

Under basal conditions approximately $10^{10}$ erythrocytes and roughly half as many granulocytes or platelets can be produced each hour, with the capacity to enhance output manifold in response to demand. This is achieved mainly through amplification of progenitors sustained by stem cell differentiation along combined stochastic and determinative pathways. Efficiency of such induced output reflects fine tuning of accessory populations in the stroma, with release of stimulatory cytokines and reciprocal negative regulators of haematopoiesis. These high generation rates and the relatively short lifespan of the more mature cells means that any compromise in normal function will have serious consequences. In aplasia, a partial or complete arrest in blood formation occurs due to loss of this regenerative capacity. However, in contrast to the pancytopenia that follows intensive chemotherapy or radiation treatment, even optimal support does not lead to marrow recovery. It has been hypothesized that such acquired irreversibility arises on the basis of heterozygosity for genes, leading to marrow failure.

Whether correct or not, environmental insults can be identified in less than half the patients and include benzene, chloramphenicol, viral infections and pregnancy.

Particularly in the pathogenesis of the idiopathic variants, mechanisms intrinsic to the stromal microenvironment have been postulated to exist on the basis of several observations. A third of the syngeneic grafts were unsuccessful without intensive preconditioning, while in one-fifth, appropriately prepared allogeneic transplants were rejected, although in some this was followed by autologous reconstitution. Furthermore, antilymphocyte globulin (ALG), used on its own or during preparation for mismatched grafting, can lead to recovery.

Further dissection of these phenomena rests on the prominence of lymphocytes amongst accessory cells of the stroma, where they participate in the control of haematopoiesis through cytokine-mediated effects on progenitors. Co-culture with peripheral blood lymphocytes derived from aplastic individuals down-regulate myeloid and
erythroid growth in clonogenic assays.\textsuperscript{14-16} The effector phenotype is E + Fcγ + OKT3 + Ia-OKM1-, and this activity is mediated through release of soluble products into the conditioned medium.\textsuperscript{17,18} The major contender for this negative regulatory function is interferon γ, which is usually secreted by these cells following lectin stimulation, but in patients, despite normal blood levels,\textsuperscript{19} it is spontaneously generated in high concentration, perhaps due to abnormal immunologic function or as a response to viral infection.\textsuperscript{20} This is seen even before sensitization to blood transfusion has taken place.\textsuperscript{21} Interestingly, in a subgroup who express autoreactive CD4 + /CD8 + immunocytes, gene rearrangement has demonstrated monoclonality for the β-chain of the T-cell receptor.\textsuperscript{22}

This line of argument is extended by demonstrating that, following haematopoietic recovery, lymphocytes that had been collected and cryopreserved before therapy with ALG were inhibitory in co-culture with autologous marrow. The suppressor effect appears to reside within the CD8 + fraction and is reversed by administering this product or antibodies to IFNγ.\textsuperscript{23} Additionally, exposure of these T-cells to ALG induces, in a dose-dependent fashion, release of several haematopoietic factors\textsuperscript{24,25} that exert antiproliferative effects on B-lymphocytes.\textsuperscript{26}

Laboratory studies have interesting clinical parallels. Thus, ALG combined with corticosteroids and anabolic androgens, correct the pancytopenia in over two-thirds of patients and in half of these blood count returns to normal.\textsuperscript{27,28} Although the mechanisms have not been clarified, response may reflect lympholysis or, as detected \textit{in vitro}, release of growth factors by accessory cells.\textsuperscript{29,30} These observations are therapeutically important in so far as the intensity of immunosuppression may determine optimal outcome, suggested in both retrospective\textsuperscript{31} and controlled clinical trials.\textsuperscript{32} Extended follow-up has shown that despite improvement in blood counts and myeloid reserve, bone marrow morphology remains abnormal,\textsuperscript{33,34} with widespread features of dysplasia and cytogenetic lesions detected.\textsuperscript{35-37} Notably, mature elements, in the form of platelets and granulocytes, were functionally defective \textit{in vitro}\textsuperscript{37} and in some, evolution to myelodysplasia, paroxysmal nocturnal haemoglobinuria (PNH) and acute leukaemia has been described.\textsuperscript{38}

When haematopoiesis in these individuals was characterised using standard bone marrow culture techniques, the clonogenic potential was found to be suppressed, consistent with reduction in the progenitor population, unbalanced interactions in the myeloid microenvironment\textsuperscript{39-42} or defective marrow stroma.\textsuperscript{43}

Attempts to explore the functional relationship between progenitors and bone marrow stroma have produced inconsistent data. Although the myeloid adherent layer appears to secrete adequate amounts of G- and GM-CSF, as well as IL-6, both constitutively and during induction with IL-1,\textsuperscript{44} some studies showed fibroblast growth to be subnormal and to support colony formation inefficiently.\textsuperscript{45-48} Similarly, monocytes and macrophages, which are prominent in the microenvironment, were also dysfunctional, and this persisted despite successful ALG therapy.\textsuperscript{49} The reported defects ranged from phenotypic disturbances to impaired production of IL-1.\textsuperscript{50,51} It is likely that these findings may well turn out to have relevance for the pathogenesis of this disease. However, data derived from long-term bone marrow cultures is not uniform (LTBMC),\textsuperscript{52,53} where irradiated stroma from aplastic patients who were responding to ALG was morphologically no different from controls and both supported equally the growth of normal progenitors.\textsuperscript{54-57} However, fewer numbers of cells expressing the CD34 antigen were detected and, predictably, had significantly diminished output on both the LTBMC and the mixed clonogenic assay.\textsuperscript{56,58,59}

These studies were extended in our laboratory, using cross-culture experiments in the blastic colony assay (CFU-b1) to exploit the adhesive properties of clonogenic cells to stroma, where
confluent layers derived from untreated patients or those responding to ALG supported colony formation to the same extent as normal controls. In contrast, stromal adherent and nonadherent aplastic precursors, selected for the CD34 antigen and cultured in a number of different assays, consistently grew less well. This population, greatly depleted of accessory cells, formed significantly fewer CFU-b1 on control stroma in a way that was unrelated to abnormalities in their adhesive properties. One interpretation is that the level of negative regulators in the aplastic stroma was not functionally higher than that of the control layers, since normal CFU-b1 formation was well supported.

In a further step, these CD34 progenitors from patients who had responded favourably to ALG were cultured and dose responses defined. It is demonstrated that low cytokine concentrations growth was poor, but with dosage escalation significant linear increments occurred, reaching control values. Such findings suggest a markedly reduced sensitivity to erythropoietin, interleukin-3 and GM-CSF.

These results contrast with earlier studies showing that when aplastic mononuclear cells were cultured in the presence of incremental concentrations of erythropoietin or GM-CSF, they were partially corrected, although this was limited to transfusion-independent patients. However, in those reports, positive selection for the CD34 population was not attempted and therefore clonogenic progenitors were cultured in unknown numbers and contaminated with accessory cells.

Studies from our laboratory have documented adequate responsiveness of aplastic precursors to the c-kit ligand when cultured in the presence of erythropoietin and IL-3. Despite suboptimal growth in the presence of more restricted stimulators, at effective concentrations, stem cell factor significantly corrected this inferior colony development. This resulted in yields not significantly different from those of control cultures, again suggesting that, in this disease, the clonogenic potential remains preserved.

Suboptimal proliferation may result from either abnormalities in the receptor densities or their affinity for cognate ligands. Additionally, corresponding soluble forms or binding proteins exist, being secreted naturally into the microenvironment or alternatively originating as proteolytic cleavage products from transmembrane molecules, that potentially may function as inhibitors to the action of their ligands in feedback regulation. However, we were able to demonstrate that clonogenic derangements in the presence of optimal concentration of growth factors ranged widely between the three cytokines, suggesting alterations in shared structures or pathways signalling for cell division.

Receptors for IL-3, IL-4, IL-7, GM-CSF, G-CSF and Epo, but not GM-CSF and c-kit, are members of a family with some structural similarities, forming part of the growing transmembrane GTP-binding group of proteins which control a series of second messenger molecules, including adenylylate cyclase, cGMP phosphodiesterase, phospholipases and ion channels. Recently, a specific 68 Kd calmodulin binding protein with nuclear localisation, common for G, GM-CSF, IL-3 and IL-6, but not for CSF-1, has been described. This glycoprotein appears to be associated with the progression from G1 to S phase of the cell cycle, and may well be important in the control of the terminal events required for the onset of DNA replication. It would be attractive to suggest that defective responses to these growth factors may have a common intracellular defect that could explain the subnormal signalling of the cytokines.

One explanation is that the lesion could be the result of persistent in vivo immune activation against target bone marrow cells, not detected by our in vitro modelling system, resulting in the morphological and functional abnormalities, the late recurrence of the disease and further response to immunosuppression. In some of these individuals the emerging population appears to remain
intrinsically abnormal, thereby predisposing them to later progression, to clonal hematopoiesis and leukemic transformation. In opposition, it could also be speculated that some of these findings in patients responding to immunosuppression, may either reflect a milder expression of the original damage to the hematopoietic stem cell or recovery of an intrinsically hyperproliferative oligoclonal escape population surviving the initial immunological attack.

Monoclonal recovery of the hematopoiesis has been described in aplasia67 and interpreted as representing a limited expansion of the stem cell pool after the first injury to the marrow, and this has similarities to that reported following intensive chemotherapy68 or experimental grafting.69 The hematopoietic progenitor cells could have been rendered kinetically disadvantaged by the preliminary insult or, alternatively, hyperproliferative clones, such as the PNH cells present in the normal polyclonal hematopoiesis that survived the attack, taking over the repopulating fraction after the damaging event.70 However, in only the minority of these patients is hematopoiesis clonal, while this is a significant feature of myelodysplastic syndromes.

These issues are of paramount importance because if the protracted autoimmune response leads to clonal evolution, considerations should be given to prescribing a more extended period of immunosuppressive therapy, administered until the reversal of the described morphological or functional markers is achieved. This approach is not unusual in patients with autoimmune haemolytic71 or ITP refractory to therapy,72 or for the treatment of graft rejection in solid organ transplantation.73

Finally, it may also be possible that the described effects happen in different clinical settings, representing virtually two different disorders, exemplified by the hypoplastic variant of myelodysplasia74 and idiopathic aplasia. Here, early discrimination between the two by molecular analysis of clonality or cytogenetics as a basis for prognosis and therapy assumes major importance.

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References
BONE MARROW APLASIA


CHAPTER 43

Hodgkin's Disease in Africa

Peter Jacobs, Werner Bezwoda, Geoffrey Falkson, and Dalila Sellami

Data from Africa regarding Hodgkin's disease is generally sketchy. This is, in part, a reflection of the priorities in underdeveloped countries, where imperatives are usually basic survival rather than the nuances of exacting disease classification. Data gathering by registries and attempts to treat lymphoma in a consistent and acceptable manner are often hampered by a shortage of resources. These range from a lack of trained staff to inadequate facilities for investigation and are aggravated by limited supplies of cytotoxic drugs or poor access to radiotherapeutic equipment.

There are vast population and cultural variations on the continent. It is thus impossible to give a comprehensive picture of differences in natural history and response to treatment of Hodgkin's disease in Africa. This is particularly true when the diverse populations, that include black, white, and those of mixed ancestry, together with others of Mediterranean stock, are considered. The available data are derived from relatively few centers, making generalization difficult.

Despite these daunting limitations, it has been possible to assemble an overview of this entity as reported during the last three decades and as presently seen by practitioners in those countries where there is access to medical records. With such observations in mind, five aspects are sufficiently circumscribed to allow more detailed analysis. Included are the issues of epidemiologic difference, if any, by race or geographic region; patterns of disease at presentation; childhood Hodgkin's disease; the association with retroviral or other infectious disease; and prevailing management programs. From this some global comments emerge.

First, there are significant differences in the pattern of Hodgkin's disease in the various populations. It appears to be less common in blacks, particularly in central Africa as well as those living in the northern and southern extremities of the continent. In addition, the mean age at diagnosis is highest in whites and lowest in blacks, with those of mixed ancestry occupying an intermediate position. Similarly, histologic subtypes vary: nodular sclerosis predominates in the Caucasian patients, whereas mixed-cellularity and lymphocyte-depleted disease are more frequent in blacks. Second, late presentation is more common among lower socioeconomic and less educated groups. This may, however, be caused more by perceptions and lack of access to medical care than by any unique or disparate disease biology. Finally, the coexistence of tuberculosis with Hodgkin's disease and the acquired immunodeficiency syndrome is widespread, thus distorting natural history and survival in this lymphoma.

Conclusions about Hodgkin's disease in Africa inevitably attract comparisons with findings in other economically underdeveloped populations, including black Americans. Such contrasts provide a chance to examine common genetic influences based on the slave routes of yesteryear and to explore those differences that may have an environmental basis. We have also had the opportunity to review experiences in the northern and southern parts of the continent and briefly to compare our findings with what is reported from other Third World countries. The conclusions are that, stage for stage, there are no fundamental disparities in regard to presentation or response to treatment. The constellation of environmentally determined adverse prognostic features that culminate in advanced stage when first seen, seem to be the major factors that impact negatively on outcome, rather than any genetically determined or ethnic differences in host or host response to the neoplasm.
The way in which these variables exert their influence leads to a perspective of poorly nourished patients who delay seeking medical attention for prolonged periods of time and, consequently, present with extensive disease. Superimposed on this is significant infectious comorbidity. Additionally, there is a lack of disciplined protocol treatment, except in a few centers where First World standards prevail. These circumstances combine to result in an outcome for this lymphoma in Africa that ranges from appallingly poor remission figures to those comparable with developed countries.

BACKGROUND

During the past three decades research in Hodgkin’s disease on the African continent has concentrated largely on epidemiology, with descriptions of clinical patterns of disease as reported in the different population groups. Pioneering studies, such as those of H. Falkson (1), focused on what was seen predominantly in white South Africans during the era before effective chemotherapy was available. Only more recently has information become available on black and other ethnic groups.

Over the last decade there has been a worldwide paradigm shift in understanding the spectrum of lymphoproliferative disorders. This was occasioned, in part, by emerging consensus in revised classification systems (2) that now include immunophenotyping and data from molecular genetics. This improved knowledge extends to Hodgkin’s disease and has led to better understanding of the entity. Wherever feasible, given the limited resources available in Africa, the newer concepts have been incorporated into classification (3,4), investigation, and treatment of these patients.

The most significant change, however, came from the introduction of effective drugs, rapidly culminating in the development of combination chemotherapy with resultant high complete remission rates. Consequently, cure is now possible in many, if not most, cases, given only that they are correctly staged and treated early. This, however, seldom happens in Africa, where a major determinant of prognosis remains high-bulk lymphadenopathy and organ involvement when patients are first seen. This phenomenon appears to be explicable by environmental factors rather than there being some unique or continent-specific difference in tumor biology. Additional adverse factors include rampant undernutrition and associated infectious disease, with human immunodeficiency virus and tuberculosis holding pride of place. To try to gain perspective on what the current situation is in Africa, five topics have been singled out for examination. In each of these a brief commentary is appended to highlight differences between data from more affluent Western patients and their counterparts from this continent. Furthermore, where possible, findings have been compared to those in blacks from other parts of the world because it is here that we have the opportunity to contrast populations with a common genetic pool but living under vastly different environmental conditions and variable levels of medical care.

EPIDEMIOLOGY WITH GEOGRAPHY AND RACE AS INDEPENDENT VARIABLES

Westernized Societies

Extensive publications, mostly from the developed or First World, show little variation in incidence when geographic areas are compared. There is a well-defined bimodal pattern of incidence and mortality with two distinct peaks evident, the first at 25 and the other at 70 years (5). These findings, which occur typically in North America, have parallels in Denmark and The Netherlands, but they are distinctly different from Japanese, Singaporean, and Indian reports.

One hypothesis advanced to explain this distribution is that the pathogenesis may differ with age. In young adults the disease has similarities to a chronic granulomatous or inflammatory process, with an extensive host reaction but paucity of malignant cells. By contrast, in the elderly, truly neoplastic behavior is more evident. This concept has precedent in the model for paralytic poliomyelitis. Here, infection with a virus of low virulence at an early age confers life-long immunity, but without such exposure, the subsequent manifestations are much more severe (6). This paradigm, however, fails to deal adequately with this lymphoma in childhood, which has a higher frequency in Africa than elsewhere, and where the predominant histologic subtype appears to be mixed cellularity with a relatively high proportion of neoplastic Reed-Sternberg cells.

An alternative hypothesis to explain these two peaks, based on Colombian studies, suggests that there is a common cause but that different presentations reflect different host and environmental factors.

African Experience

Whatever the explanation ultimately turns out to be, there are regional and socioeconomically based variations in incidence, age at presentation, distribution of histologic subtype, and therapeutic outcome when Hodgkin’s disease in different parts of the continent are compared (7–15).

Considerable geographic variation in the incidence of this lymphoma is evident in Africa, with the frequency increasing from the equator to more temperate zones. Age-standardized rates for men and women recorded in the Kampala (Uganda) Cancer Registry from 1964 to 1968 (16) were far below those from Europe and North America. Accounts from other African countries within the tropical zone are based largely on single-institution
findings from academic centers or large regional hospitals. In most of these, the prevalence of Hodgkin’s disease is low, except in childhood, when compared with other malignancies. Uniquely, a much higher incidence rate, approaching that seen in the Northern Hemisphere, was reported from the Ibadan Province of Western Nigeria (17). This may reflect intraregional variation related to undefined local factors. Relatively low frequencies are reported from Zambia (18), Kenya (19), and Zimbabwe (20). Similar data sourced from other sub-Saharan countries with cancer registries, such as Mali, Uganda, and Gambia, show figures less than 0.8 per 100,000 population (21,22).

From Algeria, based on the regional register at Sétif, rates are 2.4/100,000 in adults and 0.7/100,000 in male and 0.4/100,000 in female children, respectively (21). Tunisia has epidemiologic data from the Institut Pasteur going back to 1950 (23–25) as well as classification, clinical staging, and outcome data from 1969, when the Institut Salah Azaiz (ISA) was founded as a national cancer center (26) (Tables 1 and 2). There do not seem to be significant differences in the pattern of Hodgkin’s disease in Algeria and Tunisia, but incidence is difficult to evaluate precisely because there is no national cancer registry. The ISA figures show the disease to comprise 4% of all cancers in adults and 11% of those in children (26). In Tunisia, this lymphoma accounts for 7.4% of all cancers in both sexes (26). In Morocco the corresponding rate was 6.6% of all malignancies (27).

The pattern in the Maghreb thus appears to be that the disease affects children and young adults. Children under the age of 15 account for approximately one-third of the cases (23–27). In childhood the male-to-female ratio is approximately 3:1, whereas in adults it is greater than 2:1. Among adults, nodular sclerosing Hodgkin’s disease appears to be the most common variant. Stage distribution between stage I and II and stages III and IV is about equal. Delayed diagnosis seems relatively common, with 40% of people being seen more than 6 months after the onset of symptoms, thereby perhaps explaining the extensive disease at presentation. In children from this region, most have nodular sclerosing or mixed cellularity, with stages III and IV predominating.

In Egypt, epidemiologic data are based on studies conducted in ten different cancer centers that are attached to various universities. The major repositories for data are the Egyptian National Cancer Institute (ENCI) and the Cairo University Hospital Oncology Center, or NEM-ROCK. As a generalization, lymphoreticular tumors and leukemias in Egypt constitute between 7% and 15.9% of all malignancies. In a survey of 557 cases, high-grade lymphoma (previously designated as reticulum cell sarcoma) occurred most frequently, followed by Hodgkin’s disease. For Hodgkin’s disease, the highest incidence rate was in the second decade, and a male-to-female ratio of 3 to 1 was noted. Mixed cellularity was the most common subtype of Hodgkin’s disease, and nodular sclerosis was rare (28). In another report, differences were found comparing Egypt and the Gaza strip. In Egyptian men, lymphoma occurred more frequently than in men in the Gaza strip, whereas the inverse situation was found among women. The authors noted that this kind of comparison may provide improved approaches for discerning risk factors for cancer and advocate increased cooperation among participating countries (29).

Additional data from ENCI, analyzing a series of 4,382 newly diagnosed cancer patients, all reviewed by a single pathologist, yielded 526 cases of malignant lymphoma (7%) but only 193 cases of Hodgkin’s disease (2.57%) (30). Mixed cellularity accounted for 50.71%, lymphocyte predominant 23.78%, nodular sclerosis 17%, and lymphocyte depleted 8.29% of the patients with Hodgkin’s disease. Unfortunately, age and sex distribu
tion within the histologic variants was not recorded. In addition, at NEMROCK, 7,325 adults with cancer were reviewed between 1992 and 1995. There were 420 cases of non-Hodgkin's lymphoma (5.7%) and 107 with Hodgkin's disease (1.5%), giving a ratio of 3.8:1 (31). These data showed similar trends to the ENCI series, with 42% of patients having mixed-cellularity Hodgkin's disease. Here the male-to-female ratio was equal. Lymphocyte predominant Hodgkin's disease accounted for only 5.6% of cases with a male predominance in this subtype, and lymphocyte-depleted occurred in 7.4%, again with male predominance. Nodular sclerosis Hodgkin's disease accounted for 48 of the 107 patients over the age of 15 years, and within this subtype the male-to-female ratio was approximately 2 to 1.

Clinical staging in the Cairo University Hospital series (31) showed that 12% were in stage I, most of these without constitutional symptoms; 43% were in stage II, with half showing weight loss or other evidence of systemic involvement; 35% had stage III disease, and 10% stage IV disease: in both of the latter two categories, two-thirds to three-quarters were accompanied by systemic symptoms. Supradiaphragmatic presentation occurred in 73%, the mediastinum was involved in 20%; the liver in 30%, and only sporadic cases had disease demonstrable in bone marrow, skin, or nasopharynx (31).

The investigation of cancer patterns in migrant populations has been used as an epidemiologic tool. Migrants from North Africa to France provide useful insights into the changing pattern of lymphoreticular malignancy following relocation. When Egyptian-born settlers were compared to local-born French, with appropriate adjustment for confounding factors such as social status and areas of residence, there was a trend for higher risk of lymphoma among the migrants, although specific data on Hodgkin's disease are not available (32).

South of the equator, Hodgkin's disease in Zimbabwe shows a pattern similar to that found in North Africa, although the age split is slightly different. Approximately one-third of cases are seen before 20 years, with a male-to-female ratio of 1.8:1. A striking predominance of the lymphocyte-depleted variant was noted (Table 3).

South African data are available for the Gauteng (33) area (previously known as Pretoria and Johannesburg) and the Western Cape, and they show regional trends. Conversely, countrywide patterns are documented in the National Cancer Registry (34), which reflect a consistent pattern since the time of its establishment in 1989, with an age-adjusted rate of 0.81 for black women as compared to 1.42 for their white counterparts, and 0.95 and 3.27 for black and white men, respectively (Fig. 1). The apparently lower frequency of Hodgkin's disease in the black population may, in part, be related to underreporting because this is a pathology database. Nevertheless, these figures are consistent with that previously declared for this region (33).

| TABLE 3. Hodgkin's disease in Zimbabwe: Demographic and stage characteristics |
|-----------------------------|-------------------|-----------------|
| Number | Percent |
| Total | 170 | 100 |
| Age by decade | | |
| 0–9 | 4 | 2.4 |
| 10–19 | 48 | 28.2 |
| 20–29 | 40 | 23.5 |
| 30–39 | 37 | 21.8 |
| 40–49 | 21 | 12.3 |
| 50–59 | 8 | 4.7 |
| 60–69 | 9 | 5.3 |
| 70–79 | 3 | 1.8 |
| Sex | | |
| Male | 109 | 64 |
| Female | 61 | 36 |
| Stage | | |
| I–II | 35 | 20.6 |
| III–IV | 135 | 79.4 |
| Median age | 28 years |
| M/F ratio | 1.8 |

*LP, lymphocyte predominant; NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted. Data from L. Levy (unpublished).

There is also a marked difference between the histologic subtypes found in blacks and whites in South Africa. In the former, mixed cellularity or lymphocyte depletion predominates, whereas in the latter, nodular sclerosing is most frequently encountered. This pattern, illustrated by the Gauteng data (Table 4), also occurs in Natal and in the Freestate. The Western Cape, however, has a different population mix. In the past the population in the Cape was predominantly white or of mixed ancestry. More recently there has been a large black population influx. These population differences appear to be responsible for changes in Hodgkin's disease subtypes seen in the Cape, the pattern previously conforming to northern European data but with a significant trend toward the more frequent African pattern becoming evident since 1994.

Points of Contrast

Prominent differences are seen in the epidemiologic patterns between Africa and Europe or North America. In the terminology recommended by the International Union Against Cancer (35), Hodgkin's disease epidemiology is seen to fall into one of four categories. Type I Hodgkin's disease occurs primarily in children and is associated with less favorable histologic subtypes. Type III Hodgkin's disease predominates in developed countries and prevails in young adults, where a better outcome is associated with more frequent occurrence of lympho-
cute predominant and nodular sclerosing subtypes. Type II is intermediate between type I and type III. The type IV epidemiologic pattern is largely limited to Asia and is not discussed further here.

Viewed in this way, the type I pattern appears to be predominant in the central part of Africa (16–20). A few additional studies do deserve mention here, although in general the data were considered too sparse to be included in our main analysis of geographic pathology. A report from Zambia showed that Hodgkin's disease accounted for 18.6% of malignant lymphomas, with 44% of the cases occurring in the first two decades of life. The patients were predominantly of more advanced stage and had either mixed-cellularity or lymphocyte-depleted subtypes (18). Similar clinical presentations are reported from Nigeria (17,36–39), Kenya (19,40–42), Uganda (33,44), Gabon (45), and Zimbabwe (46–48). One set of Ugandan (44) data that may represent intraregional variation, however, describes a bimodal age-specific incidence curve approximating the type III pattern.

The pattern of Hodgkin's disease in North African littoral and South Africa is mostly intermediate or type II (20). This epidemiologic pattern approximates that seen in North America during the 1950s and 1960s (50–52), where this lymphoma occurred less frequently in blacks than whites. Hodgkin's disease in the former group had aggressive histology and was of advanced stage. These features were attributed to socioeconomic rather than genetic factors. In this regard, indications are that there has been a shift during the last 25 to 30 years among the North American black population (51) to a pattern more closely approximating type III. Based on similar inferential reasoning, one may conclude that the intermediate pattern seen in South Africa represents a transitional phase in epidemiology. However, this postulate needs to be confirmed by direct investigation to establish whether HLA or immune response–linked gene frequencies, or other genetic factors, might still play a significant role in the incidence and/or subtype distribution.

It might also be debated whether a drift in epidemiologic pattern from a lower overall frequency to a higher incidence, albeit to one with better prognostic features, represents a step forward in cancer control. It may rather be argued that available studies have not, as yet, provided any real clues to suggest a strategy of prevention of Hodgkin's disease in any population group.

<table>
<thead>
<tr>
<th>Table 4. Distribution of Hodgkin's disease by age group and histologic subtype in South Africa: Crude rates based on pathologic diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histologic Subtype</strong></td>
</tr>
<tr>
<td><strong>LB</strong></td>
</tr>
<tr>
<td><strong>NS</strong></td>
</tr>
<tr>
<td><strong>MC</strong></td>
</tr>
<tr>
<td><strong>LD</strong></td>
</tr>
<tr>
<td><strong>NDS</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

*P, lymphocyte predominant; NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted; NDS, not otherwise specified.*

Data from National Cancer Registry of South Africa (62).
CLINICAL PRESENTATION, STAGING, AND PROGNOSTIC FACTORS

Westernized Societies

The clinical features reported in North American adults and children (50–53) provide a convenient orientation against which to examine intercontinental differences. Although, predictably, attention is drawn to this entry by the finding of enlarged glands, with diagnosis dependent on node biopsy, in both developed and emerging societies there are, nevertheless, contrasting features.

African Experience

Throughout the length of this continent, the four-stage Ann Arbor classification (54–56) with modifications, as proposed at follow-up meetings in the Cotswolds (57,58), is recommended and is in general use. The problems of applying this approach to individual patients are organizational rather than methodologic. A large rural population with a limited number of centers that offer sophisticated investigations, such as computerized axial tomography, lymphangiography, and nuclear medicine techniques, have made accurate staging difficult. However, because this is a potentially curable disease, it is felt that all patients should be referred to available specialist establishments, where full and adequate investigations can be carried out. Imaging facilities are accessible along the North African littoral; in southern Africa, including Zimbabwe and South Africa; and in Kenya in East Africa, Nigeria, Ghana, and the Francophone countries.

The significant frequency of chronic bacillary and parasitic infections throughout Africa that are capable of giving rise to granulomatous or other inflammatory processes that may coexist with the lymphoma needs to be taken into account when evaluating lesions detected either clinically or radiologically (59). Such complications include, in our experience, tuberculosis, with its associated lymphadenopathy; human immunodeficiency virus infections; amebic abscesses; lymphogranuloma inguinale; hydatid cyst; syphilitic gummas; and schistosomiasis. Delay in diagnosis and empiric treatment for tuberculous lymphadenopathy, particularly among patients from rural areas, has, in our experience, been a significant cause of delay in diagnosis of Hodgkin’s disease. Although these disorders usually demonstrate features that are sufficiently distinctive for separation from Hodgkin’s disease, this does require experience and awareness of the condition.

The absence of pulmonary involvement by typical tuberculous changes should alert the clinician to the possibility that lymphadenopathy may have a cause other than this common infection. Ultimately, however, the diagnostic problem can only be resolved by adequate investigation. Access to and provision of laboratories that can make diagnoses on microbiologic and histologic grounds, rather than reliance on empiric treatment, are urgent needs in many African countries.

Enlarged spleens from endemic malarial infestation give rise to the tropical splenomegaly syndrome. Here the question of staging laparotomy and splenectomy requires judgment. The risk of malaria and other infectious diseases, on the one hand, and the fact that the majority of patients have stage IIB or more advanced illness on the other, make removal of this organ inappropriate in most of our cases. Not surprisingly, splenectomy has virtually not been used for staging in southern Africa since 1985.

Stage at presentation and prognostic features have been examined in some detail in northern and southern Africa. Data from Tunisia and Algeria (Table 2) show striking similarity, with approximately half of the patients presenting with advanced disease, B symptoms, elevated erythrocyte sedimentation rate, and a significant delay in time to diagnosis. In addition, the experience of ISA, unfortunately repeated throughout the rest of the continent, is that as many as 20% of the patients are lost to follow-up or receive inadequate treatment. The experience

TABLE 5. International Study of Prognostic Factors in Hodgkin’s Disease: Factors identified as leading to a significant reduction in failure-free survival

<table>
<thead>
<tr>
<th>Age ≤ 45 years</th>
<th>Male sex</th>
<th>Stage IV disease</th>
<th>Albumin &lt; 40 g/L</th>
<th>Hemoglobin &lt; 10.5 g/dL</th>
<th>Total WCC ≤ 1.5 × 10^9/L</th>
<th>Lymphocyte count ≤ 0.6 × 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WCC, white cell count.</td>
</tr>
<tr>
<td>Adapted from ref. 56.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. 2. Disease-free survival of chemotherapy-treated (MOPP/ABVD) white patients with Hodgkin’s disease, stratified according to the Newcastle Prognostic Index (p = .001). (Data from ref. 62.)
TABLE 6. Distribution of histologic subtype among 494 adult black and white patients with Hodgkin’s disease

<table>
<thead>
<tr>
<th>Age (decile)</th>
<th>Lymphocyte predominant</th>
<th>Nodular sclerosis</th>
<th>Mixed cellularity</th>
<th>Lymphocyte depleted</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Black</td>
<td>White</td>
<td>Black</td>
<td>White</td>
<td>Black</td>
</tr>
<tr>
<td>10–19</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>20–29</td>
<td>2</td>
<td>13</td>
<td>13</td>
<td>51</td>
<td>26</td>
</tr>
<tr>
<td>30–39</td>
<td>0</td>
<td>9</td>
<td>18</td>
<td>24</td>
<td>27</td>
</tr>
<tr>
<td>40–49</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>50–59</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>60–69</td>
<td>—</td>
<td>—</td>
<td>0</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>&gt;70</td>
<td>—</td>
<td>12</td>
<td>—</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Totals</td>
<td>7</td>
<td>36</td>
<td>58</td>
<td>171</td>
<td>83</td>
</tr>
</tbody>
</table>

Adapted from ref. 63. Overall $\chi^2 = 60.52; \ p < .001$. NS, not significant.

Striking confirmation of these differences comes from an updated study (Table 7) that showed significant differences to persist in the relative frequency distribution of components of the prognostic index, particularly histologic subtype and age, between the two populations. Despite the black patients being younger (age was a significant factor with substantial weighting toward good prognosis in the Newcastle Index), the overall outcome in these patients remained similar to that of the poor prognostic group among Caucasians. One consideration, which may be a major one in assessing the relative impact of prognostic factors on outcome of Hodgkin’s disease between blacks and whites, is that economic and educational level may be more predictive than race. However, these socioeconomic factors are correlated with race. Patient education and better socioeconomic support networks will, it is hoped, improve this situation in the future.

FIG. 3. Disease-free survival of chemotherapy-treated (MOPP or MOPP/ABVD) black patients with Hodgkin’s disease stratified according to the Newcastle Prognostic Index ($p = .1$). (Data from ref. 62.)
TABLE 7. Relative distribution of factors considered to be important in predicting outcome of Hodgkin’s disease among adult black and white patients treated with combination therapy

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th></th>
<th>White</th>
<th></th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>32</td>
<td>83</td>
<td>171</td>
<td>56</td>
<td>0.001</td>
</tr>
<tr>
<td>III and IV</td>
<td>155</td>
<td></td>
<td>136</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>29</td>
<td>11</td>
<td>95</td>
<td>31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>B</td>
<td>158</td>
<td>89</td>
<td>212</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>171</td>
<td>91</td>
<td>203</td>
<td>66</td>
<td>0.001</td>
</tr>
<tr>
<td>≥50</td>
<td>16</td>
<td>9</td>
<td>104</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥10</td>
<td>129</td>
<td>69</td>
<td>280</td>
<td>91</td>
<td>0.001</td>
</tr>
<tr>
<td>&lt;10</td>
<td>58</td>
<td>31</td>
<td>27</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Disease bulk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulky (≥10cm)</td>
<td>90</td>
<td>48</td>
<td>98</td>
<td>32</td>
<td>0.02</td>
</tr>
<tr>
<td>Nonbulky (&lt;10cm)</td>
<td>97</td>
<td>52</td>
<td>209</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.0 × 10^9/L</td>
<td>87</td>
<td>47</td>
<td>85</td>
<td>28</td>
<td>0.01</td>
</tr>
<tr>
<td>&gt;1.0 × 10^9/L</td>
<td>100</td>
<td>53</td>
<td>222</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Newcastle index</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.5</td>
<td>109</td>
<td>76</td>
<td>212</td>
<td>76</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;0.5</td>
<td>35</td>
<td>24</td>
<td>67</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

Updated from Ref. 62.

SPECIFIC ASPECTS IN CHILDREN

Westernized Societies

Childhood Hodgkin’s disease is rare in developed countries (63–66).

African Experience

Pediatric Hodgkin’s disease occurring in Egypt has been analyzed in a consecutive group of 242 children treated at the National Cancer Institute in Cairo between 1975 and 1980. There was a male predominance of 3:1. The most common histopathologic type was mixed cellularity (accounting for 60.74%). Late stages (defined as III and IV) comprised 63.2%, usually with bulky disease. Not unexpectedly, staging laparotomy (which was done in 154 cases) revealed more infradiaphragmatic disease than was clinically evident. Although an association between infradiaphragmatic Hodgkin’s disease and schistosomal hepatic fibrosis was described, this is probably no more than coincidental (67).

Currently available data from South Africa suggest that, although there may be a peak incidence between the ages of 15 and 19 in black children (34), the overall frequency is lower in blacks of all ages, including the childhood years, relative to whites (Fig. 1).

In a study from the Western Cape, including 39 children under 15 years of age, there were seven black.

TABLE 8. Childhood Hodgkin’s disease in the Western Cape: Distribution of histology by ethnic group

<table>
<thead>
<tr>
<th>Histology</th>
<th>Black</th>
<th></th>
<th>White</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>33</td>
</tr>
<tr>
<td>NS</td>
<td>2</td>
<td>29</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>LD</td>
<td>3</td>
<td>43</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>LP</td>
<td>1</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>14</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>20</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*MC, mixed cellularity; NS, nodular sclerosis; LD, lymphocyte depleted; LP, lymphocyte predominant.

Data from ref. 71. Nodular sclerosing was present in 59% of white patients, whereas mixed cellularity dominated in those of mixed ancestry at 40%, and lymphocyte depletion in blacks at 43%. Mixed cellularity was present in 37% of the children under 11 and 25% of the older group, while lymphocyte depletion occurred in 30% of the older group and only 12% of the younger cases. The incidence of nodular sclerosing histopathology in whites and those of mixed ancestry did not achieve significant differences, again probably because of small numbers.
TABLE 9. Childhood Hodgkin’s disease in the Western Cape: Distribution of stage by ethnic group

<table>
<thead>
<tr>
<th>Stage</th>
<th>Whole group</th>
<th>Black</th>
<th>Mixed</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>41</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>28</td>
<td>2</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>26</td>
<td>3</td>
<td>42</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>7</td>
<td>20</td>
<td>12</td>
</tr>
</tbody>
</table>

Data from ref. 71.

White and 20 children of mixed ancestry (68-70) (Tables 8 and 9). The male-to-female ratio was 2.9:1, and the median ages were 147, 124, and 119 months in children of white, mixed, and black ancestry, respectively. The latter two groups came mainly from a poor socioeconomic background. Systemic symptoms were present in 51% of cases. Histologic subtypes included nodular sclerosis in 35% of white patients, mixed-cellularity in 40% of mixed ancestry patients, and lymphocyte-depleted in 43% of black patients. Five percent of the entire group had clinical stage I; 41% stage II; 28% stage III; and 28% stage IV disease. By contrast, the majority of white children presented with stages I and II.

Points of Contrast

A recent study in children (<13 years old) from Johannesburg (62) emphasizes the Capetown data (Table 10). Notably, in this group of 91 patients, of whom 61 were black and 30 were white, there was a clear demonstration of a difference in distribution of prognostic factors, with late-stage disease and lower hemoglobin levels among black children as compared to their white counterparts.

Similar observations were noted in Namibia (71). Significantly, here, the increased risk of developing tuberculosis was sufficient to lead to the suggestion that

TABLE 10. Clinical and pathologic features among 91 children with Hodgkin’s disease from Johannesburg (Gauteng)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Black</th>
<th>White</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>47</td>
<td>22</td>
<td>.01</td>
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<td>Female</td>
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Histologic subtype:

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<th>p Value</th>
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<tbody>
<tr>
<td>LP</td>
<td>1</td>
<td>3</td>
<td>0.01</td>
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<tr>
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<td>20</td>
<td>12</td>
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<tr>
<td>MC</td>
<td>39</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>LD</td>
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<td>1</td>
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Stage:

<table>
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<tr>
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<th>White</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I and II</td>
<td>18</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>III and IV</td>
<td>43</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

Symptoms:

| A | Black | 25 | 27 | 20 | 21 | <.03 |
| B | Black | 36 | 40 | 10 | 12 | <.03 |

Hemoglobin:

| < 10 | Black | 24 | 26 | 18 | 20 | <.03 |
| < 10 | Black | 37 | 41 | 12 | 13 | <.03 |

Lymphocyte count x 10^6/L:

| < 1.0 | Black | 50 | 55 | 18 | 19 | <.03 |
| < 1.0 | Black | 11 | 12 | 12 | 12 | <.03 |

Disease bulk:

| Bulky | Black | 31 | 34 | 5  | 5  | <.03 |
| Nonbulky | Black | 30 | 33 | 25 | 27 | <.03 |

Age:

| Mean ± SD | Black | 8.2 ± 3.1 | 9.4 ± 4.1 |

Data from ref. 63.

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prophylactic anti-TB therapy is appropriate in all children with malignancy being treated in the developing countries of Africa (72).

VIRAL INFECTION AND HODGKIN’S DISEASE

Epstein-Barr Virus

Westernized Societies

Numerous studies have examined the role of Epstein-Barr virus (EBV) (73,74) in Hodgkin’s disease. Although initial serologic studies were unable to show any clear clinical relationship between this lymphoma and the virus, the advent of in situ hybridization studies revealed an integrated genome in the Reed-Sternberg cells in 35% of cases (75). Furthermore, at least one gene product, the latent membrane protein (LMP-1), can be found on the surface of the multinucleate tumor cells. The LMP may function as a target for cytotoxic T lymphocytes and thereby facilitate host control over the neoplasm. Conversely, if this surveillance mechanism is lost, the same molecule appears to have the capacity to enhance proliferation of the infected cells, leading to the emergence of a histologically aggressive tumor (73–75). These apparently opposing effects might underlie differences in the course of this lymphoid malignancy and raise the interesting possibility that prevention may be achieved by means of vaccination.

African Experience

The role of Epstein-Barr in Hodgkin’s disease occurring in different geographic areas has been investigated in a number of studies (76,77). Differences in the frequency of expression of EBV in neoplastic cells of Hodgkin’s disease have been noted when cases from Kenya and Italy were compared (78). In another study biopsy material from cases of childhood Hodgkin’s disease occurring in ten different countries were compared: LMP-1 was found in 50% to 100% of cases, with the highest rates of expression tending to occur in cases from underdeveloped countries. By a sensitive polymerase chain reaction–based EBV strain–typing procedure (76), EBV strain type I was shown to be predominant in childhood Hodgkin’s disease from the United Kingdom, South Africa, Australia, and Greece; EBV strain type II was predominant in Egypt. Both EBV strain types I and II were detected in some cases of childhood Hodgkin’s disease from the United Kingdom, Costa Rica, and Kenya, with the frequency of dual infection being highest in cases from developing countries. The authors speculated that the high incidence of EBV and the presence, especially in developing countries, of dual infections with both type I and type II may reflect socioeconomic conditions leading to malnutrition–induced immunologic impairment (76).

The Human Immunodeficiency Virus

Westernized Societies

The possible role of the human immunodeficiency virus (HIV) in Hodgkin’s disease is attracting increasing attention. A number of recent publications from Europe and the United States have suggested an association between the two (79–85). In this setting, at-risk individuals are homosexual men with HIV disease and/or intravenous drug abusers with low CD4 counts and with significant evidence of impaired immunologic integrity.

African Experience

High rates of immunodeficiency viral infection and clinical AIDS occur throughout Africa. A causal relationship could be expected to have a major impact on Hodgkin’s disease incidence. HIV seropositivity rates of approximately 7% to 10% have been recorded among antenatal clinic attendees in South Africa (86). Even higher figures have been reported from Central Africa.

However, no increased incidence of Hodgkin’s disease has emerged in cancer registry data. In a recent case-control study involving 913 blacks with malignant disease (87) conducted in Johannesburg, a notable correlation between HIV infection and neoplasia was observed only for Kaposi’s sarcoma, with 27 of 35 patients being seropositive (odds ratio 61.8, 95% C.I. 19.7 to 194.2), and for non-Hodgkin’s lymphoma (27 of 40 individuals seropositive for HIV, with an odds ratio of 4.8, 95% C.I. 1.5 to 14.8). This association is similar to that noted in several other sub-Saharan African communities (88–90), where HIV viral prevalence is high. The odds ratio for the association of HIV and non-Hodgkin’s lymphoma was, however, lower than that reported in developed countries. The reasons for these findings are not clear but may include early mortality from tuberculosis in African HIV cases. This infectious complication occurs at higher CD4 counts (about 300 to 400/μL) counts than those associated with the development of non-Hodgkin’s lymphoma. No other cancer observed, including Hodgkin’s disease, as well as those arising from liver, vagina, penis, esophagus, cervix, or the oropharynx (all of which may have an infectious etiology), showed a significant relationship to infection with HIV. The Johannesburg study showed an HIV seropositivity rate of 10.8% in patients with Hodgkin’s disease, giving an odds ratio of 2.0 (95% C.I. 0.6 to 6.6).

Although there was no notable correlation between HIV and Hodgkin’s disease, the coincidental occurrence of the two entities does exist, particularly because both occur in younger patients. Of the 37 patients with Hodgkin’s disease who were HIV positive, clinical data were available for 28. The identification of these individuals allowed some observations to be made regarding the clinical aspects of Hodgkin’s disease with HIV in black
TABLE 11. Presenting clinical and laboratory features in 28 black HIV-positive patients with Hodgkin’s disease

<table>
<thead>
<tr>
<th>Histologic subtype*</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>MC</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>LD</td>
<td>7</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>19</td>
<td>68</td>
</tr>
<tr>
<td>V</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>Male</td>
<td>18</td>
<td>64</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>36</td>
</tr>
<tr>
<td>Bulky disease (&gt; 10 cm)</td>
<td>11</td>
<td>39</td>
</tr>
<tr>
<td>Nonbulky disease (&lt;10 cm)</td>
<td>17</td>
<td>61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hemoglobin (g/dL)</th>
<th>10.3 ± 1.2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes (×10^9/L)</td>
<td>0.849 ± 0.102b</td>
</tr>
<tr>
<td>Age (years)</td>
<td>20.9 ± 4.2b</td>
</tr>
</tbody>
</table>

*NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted.
*bMean ± SD.

TREATMENT OUTCOMES

Westernized Societies

Evidence from an expanding literature clearly demonstrates that Hodgkin’s disease is curable. Cardinal determinants are reliable diagnosis, accurate staging, and management with appropriate and well-tested multimodality programs that comprise combination chemotherapy alone or combined with irradiation (91-93).

There has been a major shift from the early days when clinical assessment was converted to pathologic staging by surgery (94,95). Currently favored are less invasive methods that center on technological advances, including high-resolution imaging procedures (96,97).

Despite the favorable outcome for most patients treated with conventional approaches, there are still a number of problems. The first is refractory, slowly responding, or relapsed disease, which does better with high-dose chemotherapy and myeloprotection using peripheral blood hematopoietic stem and progenitor cells (98-100). Equally important is the ever-increasing appreciation that, although conventional treatment has curative capacity in sensitive patients, late complications arise and adversely affect outcome. Safer but equally effective regimens are needed (101-102).

African Experience

Treatment has been modified to keep abreast of new advances. In summarizing contemporary practice, radia-

TABLE 12. Hodgkin’s disease and HIV in Zimbabwe: 89 patients of known HIV status

<table>
<thead>
<tr>
<th>HIV positive</th>
<th>HIV negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td>Histologic subtype*</td>
<td></td>
</tr>
<tr>
<td>LP</td>
<td>0</td>
</tr>
<tr>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>MC</td>
<td>24</td>
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<tr>
<td>LD</td>
<td>6</td>
</tr>
<tr>
<td>Age</td>
<td>34</td>
</tr>
<tr>
<td>Median</td>
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<td>Range</td>
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<table>
<thead>
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<td>I-II</td>
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<td>16</td>
</tr>
<tr>
<td>III-IV</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Sex</td>
<td>M/F ratio</td>
<td>3.7:1</td>
</tr>
<tr>
<td></td>
<td>1.3:1</td>
<td></td>
</tr>
</tbody>
</table>

*LP, lymphocyte predominant; NS, nodular sclerosis; MC, mixed cellularity; LD, lymphocyte depleted; M, male; F, female.

Data from L.M. Levy (unpublished data).
Radiation and chemotherapy have been somewhat artificially isolated, and children are considered separately.

Adults: Radiation Therapy

In a report from Johannesburg (62), the outcome after laparotomy, for stage I to IIIA Hodgkin's disease treated between 1976 and 1986 with total nodal irradiation, was much poorer for black patients than for whites. However, in this retrospective analysis, when patients with stage I disease and those with nonbulky stage II disease with normal hemoglobin levels were considered, the outcome in blacks did not differ from that in whites with corresponding early stage similarly managed. These results suggest that radiation treatment can play a significant role on this continent, as elsewhere, provided diagnosis is prompt and referral appropriate. One problem is, however, access to radiation therapy centers. To this end, the World Health Organization, in association with the International Atomic Energy Commission, has embarked on a project of installing the necessary equipment and providing oncology training.

Adults: Chemotherapy

Systemic treatment is based on the traditional MOPP combinations. With few exceptions, the response rates in blacks emerge as significantly poorer than those observed with similar treatment regimens in other parts of the world (103–106).

From the evidence available, patients with unfavorable histology and advanced disease have a better outcome following six or even eight cycles of multidrug therapy combining MOPP, or its variant, with one or another form of ABVD than following MOPP alone (107–111). A significant factor may be the higher dose intensity achieved with the hybrid regimens (112–114). There have, however, been no randomized studies to determine whether this observation applies equally to all population groups. Our own experience, based on a retrospective analysis, is that there may be a trend toward improvement in blacks when the MOPP-ABVD era (1985 to 1995) is compared to earlier experience (1970 to 1983), when MOPP alone was used (Figs. 2 and 3). However, despite some improvements, the discrepancy between black and white individuals with Hodgkin's disease, equivalently treated, remains evident (Fig. 4). Costs are also significantly higher for regimens containing anthracycline, such as ABVD.

Data from the Cairo University Center are instructive. The overall and disease-free survival is shown in Figure 5. Survival appears, in general, to be inferior by 10% to 15% to that reported for Western series except for those cases with stage I disease, treated with mantle or inverted-Y radiation therapy, where survival exceeds 88% at 5 years. The corresponding figures are 48% for stage II, 39% for stage III, and 33% for stage IV. As elsewhere, the explanation may be related to irregular treatment courses, with 47% being delayed more than 2 weeks because of social circumstances (31).

A significant dose–response relationship has been shown for Hodgkin's disease with favorable results for high-dose chemotherapy linked to autologous bone marrow transplantation for patients with recurrent disease (115–116).

Because both dose intensification and better treatment compliance might be achieved using high-dose chemotherapy with autologous bone marrow support for advanced poor prognosis disease, a trial was initiated in Johannesburg using this as the initial approach. Twenty-six patients are currently evaluable. The regimen consists
of melphalan (140 mg/m² IV) combined with etoposide (VP16, 2.5 g/m²). Hematologic rescue is effected using noncryopreserved autologous marrow (four patients) or G-CSF-stimulated peripheral blood stem and progenitor cells (22 patients). Not having to freeze and store the rescue products has made the whole procedure technically simpler and cheaper. All patients have reconstituted (to ≥1.0 × 10⁶/L neutrophils and ≥40 × 10⁶/L platelets, without transfusion dependency) with median recovery time of 17 days. Median hospitalization time was 19 days.

Twenty-four of the 26 patients achieved complete remission following one cycle of treatment. Only one patient had a partial response, and one failed, giving an overall complete response rate (24 of 26) of 92%. The first six patients in this study underwent only a single course of high-dose chemotherapy. There were three recurrences (at 18, 22, and 25 months, respectively) (117). All subsequent patients had the induction cycle repeated and were autografted (as outlined above) with the second cycle, administered 4 to 6 weeks after the first procedure. Time to hematologic recovery was not significantly different following the second course of therapy in comparison to the first. Among the 20 patients given double high-dose chemotherapy with peripheral blood stem-cell rescue, the complete remission rate was 100%. At a median follow-up of 30 months, there have been no recurrences among the patients who were treated twice (118). The disease-free survival for all 26 patients is shown in Figure 6.

Although this is not a randomized study, it is of interest to note that the single cycle of chemotherapy has an adequate cure rate, even though the disease is chemotherapy sensitive. The efficacy of a double induction cycle remains to be established on further follow-up. However, if successful, this approach is likely to provide an acceptable method because it is additionally associated with a reduction in total treatment time for patients with poor prognostic Hodgkin's disease in Africa.

Children

Although reported series are small, results suggest an outcome superior to that achieved in adults (119,120). Exceptionally good results have been reported with MOPP for childhood disease in Africa with initial complete response rates of 85% to 100% (67,68,121,122). This outcome is noticeably better than that achieved in adults using the same therapeutic regimen. In a series reported from the Tygerberg Academic Hospital, those under 15 years of age (Tables 8 and 9) treated with MOPP, or its equivalent, CHIVPP, or the MOPP/ABVD hybrid together with 20 to 30 Gy involved-field radiotherapy to bulky mediastinal disease (70) had a projected survival at 10 years of 85% for stage I and II disease and 82% and 48% at 5 and 10 years, respectively, for stage III and IV disease (Fig. 7). Survival in children was identical, irrespective of chemotherapy used (Fig. 8). Disease- and treatment-related complications were, however, frequent. Of the infections, tuberculosis was common in the Western Cape and caused significant morbidity; one-third of the patients required antituberculosis treatment.
CONCLUSION

Hodgkin's disease, as seen among children and adults throughout Africa, creates a sense of déjà vu among investigators working in First World centers. Our understanding of the available evidence is that there is a single causative pathophysiologic process, but the impact of environmental factors is much more profound than in the more uniform societies generally evolved in the Western World. Of these, the presentation and clinical course are profoundly and adversely influenced by malnutrition and by rampant and escalating human immunodeficiency disease positivity, with its linked epidemic of tuberculosis. Logic would dictate that, if these compounding factors could be corrected, little difference would be seen either between blacks and whites on the continent or in comparison with more affluent societies in other parts of the world. On currently available evidence, these appear to be unobtainable and even receding goals. Perhaps the most frightening reality to be faced is a continent-wide deterioration in medical standards, with reduction and restriction of available resources that range from unavailability of chemotherapy drugs to the nondelivery of radiation and radiation therapy equipment. If this is the truth, as seems to be the case, Africa will continue to provide a model for the study of differences between affluent and poor. This is a chilling scenario and the antithesis of what all dedicated doctors strive toward. The goal must be improvement in the investigation and management of these individuals. Only in this way can the outcome be elevated to levels that in the future will differ in no significant way from the standard-setting academic centers in the First World.

ACKNOWLEDGMENTS

The authors wish to thank many colleagues in Africa who provided data for this chapter. Included are Lorraine Levy, Associate Professor, Department of Medicine, at the University of Zimbabwe and the Zimbabwean Cancer Registry; Dr. Handy Azim, of the Cairo Oncology Center; Peter Hesseling, Professor and Head of the Department of Paediatrics and Child Health, University of Stellenbosch, who was particularly helpful in providing additional data from his own experience and records; Gary Culligan, Professor, Medical University of Southern Africa; Coenrad F. Slabber from the Department of Medical Oncology, University of Pretoria; Seditia Isaacs from the University of Cape Town, Groote Schuur Hospital; and Pauline Close, Department of Anatomical Pathology, University of Cape Town and Groote Schuur Hospital. We would also like to thank Christine Dölling for providing invaluable bibliographic assistance; and Deirdre Collins and Gillian Ganz, who prepared and typed drafts and the final manuscript.

REFERENCES

43: Hodgkin's Disease in Africa / 767


48: HODGKIN’S DISEASE IN AFRICA / 769


Effect of age on the characteristics and clinical behavior of non-Hodgkin’s lymphoma patients

The Non-Hodgkin’s Lymphoma Classification Project*

*See page 977 for list of study participants

Summary

Background: The goals of this study are to describe the frequency, clinical characteristics, and outcome of the different non-Hodgkin’s lymphomas according to age.

Patients and methods: Patients included in the recently published analysis of the Non-Hodgkin’s Lymphoma Classification Project were analyzed. All patients had their slides reviewed and classified by five independent expert hematopathologists. Lymphomas were classified according to the Revised European–American Classification of lymphoid neoplasms. Sufficient data were available on 1283 cases. Five age groups were analyzed: < 35 years, 35–49 years, 50–59 years, 60–69 years, and ≥ 70 years.

Results: Few differences were observed between the age groups with regard to lymphoma types and clinical characteristics. Anaplastic large cell lymphoma, Burkitt’s lymphoma, and lymphoblastic lymphoma were observed more frequently in patients younger than 35 years, whereas small lymphocytic and lymphoplasmacytoid lymphomas were observed more frequently in patients older than 70 years. Mantle cell lymphoma and marginal zone lymphomas were observed more frequently in middle-aged patients. Poor performance status was more frequent in older patients, as was bone marrow infiltration, whereas spleen involvement was more frequent in younger patients. Young and older patients had a slightly worse age-adjusted International Prognostic Index score (P < 0.01). Complete response rates decreased with age from 68% in the youngest patients to 45% in the oldest patients (P < 0.0001). Median event-free survival and overall survival also decreased with age (P < 0.0001).

Conclusions: Elderly patients have a poorer outcome than younger patients but age alone is not sufficient to discriminate patients with a poor outcome. However, the histologic type of lymphoma and clinical characteristics may define a subgroup of patients with a poor outcome in each age category.

Key words: age, elderly patients, non-Hodgkin’s lymphoma

Introduction

Although the incidence of non-Hodgkin’s lymphoma (NHL) is steadily increasing, particularly in elderly patients [1–3], few studies have described the histologic and clinical characteristics of the elderly patients. In some diseases, such as diffuse large cell NHL, it is known that older age is associated with a poor outcome, and that elderly patients frequently have more clinical features associated with a poor prognosis, such as poor performance status (PS), B symptoms, or concomitant diseases [4–8]. Some lymphomas, such as mantle cell lymphoma, have been reported to occur more commonly in elderly patients, while other lymphomas, such as Burkitt’s lymphoma or mediastinal B-cell lymphoma, occur less often in older patients. While the relative frequency of the various diseases, as described in the recently presented Revised European–American Lymphoma (REAL) Classification [9], was recently documented [10] no analysis were for the different classes of age, nor have patient characteristics and outcomes been addressed in relation to their age.

Therefore, we have used the 1403 patients previously reported in a clinical evaluation of the REAL Classification [10] to describe the frequency of each lymphoma, the clinical characteristics, and the outcomes according to the age of the patients.

Patients and methods

The patients included in this analysis were described in the previous paper reporting this study [10]. Briefly, nine institutions in eight countries provided up to 200 consecutive cases of previously untreated NHL beginning from 1 January 1988. In all cases, tissues biopsies had to be adequate for diagnosis and classification using the REAL Classification, including immunophenotyping. Five expert hematopathologists (J. Diebold, H.K. Müller-Hermelink, K. MacLennan, B.N. Nathwani, D.D. Weisenburger) reviewed all cases. Clinical characteristics, treatment data, and follow-up were required in all cases. The clinical information for each case was abstracted from the medical record by a clinician or data manager and recorded on a standardized form. These data included coded patient and site identifiers, sex, date of birth, date and site of diagnostic biopsy, a tabulation of nodal and extranodal sites of disease, Ann Arbor stage, performance status (PS), and maximum diameter of largest tumor mass at diagnosis. Laboratory data were recorded including serum lactate dehydrogenase (LDH) level, absolute lymphocyte count, presence of circulating lymphoma cells, presence of a monoclonal immunoglobulin, a history of immunodeficiency, and viral (HTLV-I, HIV) status. The initial therapy and therapeutic response, details of remission, progression, or relapse, and subsequent therapies and follow-up were tabulated for each case. Treatment was coded using the following criteria: unknown therapy,
no treatment given initially; radiation therapy only, surgery only, single agent chemotherapy (e.g., chlorambucil), CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) or CHOP-like (containing cyclophosphamide and doxorubicin at the same dosage than the CHOP regimen plus other drugs) regimens, high-dose CHOP regimens (with doxorubicin administered at ≥ 70 mg/m² and cyclophosphamide at ≥ 1050 mg/m² plus other drugs).

One thousand four hundred and three cases were entered in this study. Twenty-five cases were found to have a diagnosis other than lymphoma after review and, thus, were excluded from further analysis. Of the 1378 remaining cases, 95 had incomplete clinical data or follow-up data and were not included in this analysis, leaving 1283 cases. To create homogeneous groups with approximately the same number of patients, five classes of age were chosen (Table 1): less than 35 years old, 35 to 49 years, 50 to 59 years, 60 to 69 years, 70 or older. Even with that the percentage of patients in the oldest groups were higher than in the younger groups.

Completed clinical and pathology forms were reviewed and edited to detect any inconsistencies, and additional information and/or clarification was obtained when needed. After completion of the editing, the clinical and pathology forms were entered into a computer for data analysis [10]. The International Prognostic Index [11] was used to stratify patients with various disease entities. Treatment outcome was measured using event-free survival and overall survival. Event-free survival was defined as the time from diagnosis to the first occurrence of progression, relapse after response or death from any cause. Follow-up of patients not experiencing one of these events was censored at their date of last contact. Overall survival was measured from diagnosis to death from any cause, with surviving patient follow-up censored at the last contact date. Estimates of event-free survival and overall survival distribution were calculated using the method of Kaplan and Meier [12]. Time to event distributions were compared using the log-rank test [13].

### Results

The distribution of the different histologic subtypes according to age of the patients at diagnosis is shown in Table 1. Anaplastic large-cell lymphoma (ALCL), Burkitt's lymphoma (BL), and lymphoblastic lymphoma (LL) were observed mostly in young patients (less than 35 years old). Mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), and mucosa-associated lymphoid tissue (MALT) lymphoma were observed more frequently in middle aged patients (35 to 69 years). Small lymphocytic (SLL) and lymphoplasmocytoid lymphoma (LPL) were observed predominantly in older patients. Follicular lymphoma (FL), diffuse large B-cell lymphoma (DLCL), and peripheral T-cell lymphoma (PTCL) patients had a distribution comparable with the whole population, that is an incidence slightly increasing with age. Nevertheless, DLCL and FL remained the two most frequent lymphomas in all age categories, representing respectively 30% to 40% and 20% to 30% of all lymphomas by age category. SLL/LPL, MALT, and PTCL only represented between 5% and 10% of all lymphomas, and the other lymphoma types less than 5% in all age groups.

Table 2 shows the main clinical characteristics of these patients and the most frequent extranodal sites according to age distribution. The youngest patients were predominantly male and oldest patients predominantly female ($\chi^2 = 16.5, P < 0.005$). While poor

---

**Table 1. Frequency of different lymphomas in the 1283 patients according to age.**

<table>
<thead>
<tr>
<th>Histologic subtypes</th>
<th>Number of pts</th>
<th>Percentages of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small lymphocytic / lympho-plasmacytoid lymphoma</td>
<td>98</td>
<td>14</td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>105</td>
<td>8</td>
</tr>
<tr>
<td>Marginal zone lymphoma (spine and nodal)</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>317</td>
<td>8</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>72</td>
<td>11</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>448</td>
<td>16</td>
</tr>
<tr>
<td>Peripheral T-cell lymphomas</td>
<td>93</td>
<td>11</td>
</tr>
<tr>
<td>Anaplastic large T-cell lymphoma</td>
<td>32</td>
<td>53</td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>9</td>
<td>78</td>
</tr>
<tr>
<td>Lymphoblastic lymphoma</td>
<td>28</td>
<td>68</td>
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<tr>
<td>Unclassified</td>
<td>46</td>
<td>6</td>
</tr>
<tr>
<td>All patients</td>
<td>1283</td>
<td>13</td>
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</tbody>
</table>

**Table 2. Clinical characteristics according to age.**

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Number of pts</th>
<th>Percentages of patients</th>
</tr>
</thead>
</table>
| Sex                      |               | 35-49 | 50-59 | 60-69 | 70+
| Male                    | 662 | 64 | 51 | 50 | 53 | 45 |
| Female                  | 621 | 36 | 49 | 50 | 47 | 55 |
| Stage                    |               | 35-49 | 50-59 | 60-69 | 70+
| I                       | 238 | 22 | 17 | 15 | 20 | 22 |
| II                      | 259 | 28 | 18 | 21 | 20 | 19 |
| III and IV              | 746 | 50 | 65 | 64 | 60 | 59 |
| Poor performance status | 234 | 20 | 14 | 17 | 14 | 30 |
| More than one extranodal site | 396 | 29 | 33 | 24 | 29 | 30 |
| Tumor larger than nine cm | 295 | 26 | 23 | 26 | 23 | 30 |
| Above normal LDH value | 518 | 47 | 37 | 47 | 49 | 47 |
| Bone marrow involvement | 417 | 15 | 35 | 38 | 36 | 30 |
| Splenic localization     | 229 | 13 | 20 | 24 | 18 | 14 |
| Gastrointestinal localization | 178 | 14 | 12 | 12 | 14 | 15 |
| Head and neck localization | 122 | 12 | 8 | 11 | 7 | 10 |
| Skin localization        | 104 | 7 | 7 | 7 | 9 | 9 |
| Liver involvement        | 103 | 38 | 11 | 10 | 7 | 5 |
| Pleural involvement      | 73 | 7 | 6 | 7 | 5 | 4 |
| Lung involvement         | 54 | 5 | 3 | 4 | 5 | 4 |
| Bone involvement         | 38 | 3 | 4 | 3 | 3 | 4 |
| CNS involvement          | 33 | 5 | 3 | 2 | 2 | 2 |
| Orbit localization       | 17 | 2 | 1 | 2 | 1 | 2 |
| Thyroid localization     | 10 | 10 |
| Testicular                | 8 | 7 |

*All parameters were not known for each patient.*
involved in 33% to 4% of the patients in decreasing order of frequency (Table 2). Bone marrow involvement was observed in only 19% of the youngest patients compared to 30%-36% in older patients ($\chi^2 = 20.8$, $P < 0.0005$). Spleen involvement was observed less frequently in young and old patients (13% and 14% compared to 17%-24% for intermediate age, $\chi^2 = 12.9$, $P < 0.02$) and thyroid involvement was observed essentially in oldest patients (2.4% compared to <0.5%, $\chi^2 = 15.2$, $P < 0.01$). For all other sites of involvement there were no statistically significant differences among the different age groups. In summary, except for bone marrow involvement and poor PS, very few differences were observed among the different age groups.

Table 3 shows the distribution of patients by age group according to the International Prognostic Index (IPI) scores [11], index based on age ($\leq 60$ or $> 60$ years), stage (localized or disseminated), PS (0-1 or $\geq 2$), number of extranodal sites (0-1 or $\geq 2$), and LDH level (normal or increased); and according to the Age-Adjusted Prognostic Index (AAPI), index based on stage, PS, and LDH level. The IPI was largely influenced by the age of the patients: 60% of patients younger than 60 years had a good score and less than 20% a bad score, compared to 25% and 50% for patients older than 60 years, respectively ($\chi^2 = 224.2$, $P < 0.00001$). For the AAPI, the distribution of patients in each score was more homogeneous: slightly more youngest and oldest patients had a worse score compared to intermediate age patients ($\chi^2 = 28.8$, $P < 0.005$).

Table 4 shows treatments given to these patients, the response rates to these treatments, and the progression rates according to the age of the patients. Because of the heterogeneity of treatments, it is not possible to correlate response rates to the different types of treatment. The percentage of patients treated with CHOP-like or high dose regimens decreased with age. This may reflect in part the higher percentage of patients with SLL, however, predominantly, it is due to the natural tendency of physicians to decrease the intensity of the treatment in older patients since they have poor PS or associated diseases. Less than 40% of the patients with SLL, MZL, or MALTL were treated with anthracycline-containing regimen. No initial treatment was the choice at diagnosis for patients with MZL (28%), SLL (23%), and MALTLymphoma (13%) patients; in contrast, only very few patients had no initial therapy for other lymphomas (8% of FL, 6% of DLCL and PTCL, 5% of MCL patients). Surgery only was used in 10% of MALTLymphoma and 6% of MCL patients. Radiation therapy only was used in 12% of FL, 11% of BL, and 11% of MALTLymphoma patients. Complete response (CR) at the end of treatment decreased with age from 68% in youngest patients to 45% in oldest patients ($\chi^2 = 62.9$, $P < 0.0001$) accompanied by an increasing partial response (PR). However, as shown in Table 4, treatments differed with age groups and this decrease may witness either the refractoriness of the lymphoma or the inefficacy of the treatment.

Disease progression was observed slightly less frequently in the youngest patients ($\chi^2 = 10.1$, $P < 0.05$), but true relapse rate from CR did not increase with age (Table 4). Median event-free survival and overall survival as well as the percentages of patients free of disease or alive at three years or five years decreased with age (Table 4 and Figure 1). The number of patients who reached a CR then died in CR from a concomitant disease is more important in patients older than 70 years (8%) compared to 2%-5% in younger patients.

Patients with the most common lymphomas, diffuse large B-cell lymphoma, had a survival pattern that did not differ from that described for the entire study population, with median survival and event-free survival
Figure 1. Survival of the patients included in the International Non-Hodgkin's Lymphoma Classification Project according to their age at diagnosis ($\chi^2 = 80.87, df = 4, P < 0.0001$).

Figure 2. Overall survival of follicular lymphoma patients included in the International Non-Hodgkin's Lymphoma Classification Project according to their age at diagnosis (log-rank test, $\chi^2 = 23.95, df = 4, P < 0.0001$).

Figure 3. Overall survival of diffuse large B-cell lymphoma patients included in the International Non-Hodgkin's Lymphoma Classification Project according to their age at diagnosis (log-rank test, $\chi^2 = 42.72, df = 4, P < 0.0001$).

Figure 4. a) Overall survival according to the age-adjusted International Prognostic Index for patients younger than 60 years and with known parameters. b) Overall survival according to the age-adjusted International Prognostic Index for patients 60 years or older and with known parameters.

1108 patients had a known value for the age-adjusted IPI (Table 3). Event-free survival and overall survival for the whole group of these patients reflected that described for aggressive lymphoma patients (data not shown). Patients younger and older than 60 years had the same pattern of outcome according to the AAPI, but younger patients had an always slightly better outcome in all AAPI scores (Figure 4). Figure 4b clearly shows that elderly patients may have a good or a poor outcome in relation to the number of adverse prognostic parameters as described in the AAPI.

Discussion

This series of 1283 patients is the first with a modern description of histologic subtypes and a description of standard prognostic factors particularly for a comparison according to patient's age. As was pointed out recently, clinical prognostic factors are as important as the histologic subtype for proper management of patients [10, 14]. We confirm that age was one of the most important prognostic factors in diffuse large cell lymphomas [4, 5, 7, 11, 15, 16]. Our analysis confirm that age is also an important prognostic factor for response to treatment, event-free survival, disease-free survival, and
overall survival for DLCL patients; in addition, the importance of age is also applicable for all lymphoma subtypes. Why older age has an adverse effect on patient outcome is not completely understood: a low response rate and an increase in death rate may be due to the presence of poorer conditions in the elderly, mostly because of comorbid conditions [4–8]. In fact, 8% of the oldest patients died in CR from another disease compared to less than 5% in all the other age groups. Although, a decrease in immune surveillance has been suggested as a factor responsible for this poorer outcome, direct evidence of this defect as a contributory factor has not been published.

The increased proportion of some lymphomas at different ages has already been described, however our analysis shows some new findings. Nearly all lymphomas may be observed in any age category but SLL/LPL, MALT lymphoma, MZL, MCL, and PTCL are rare in patients under 35 years, whereas ALCL, BL, and LL are rare in patients over 70 years (Table 1). The preponderance of young patients with lymphoblastic and Burkitt’s lymphomas is confirmed in this study, but these lymphomas may also be observed in elderly patients. MCL was initially described in older patients, but can in fact be observed in patients younger than 60 years. These observations lead us to believe that even in the elderly patients any lymphoma can occur and that accurate histologic diagnosis is essential since different outcomes are observed for the different diseases and these patients require different treatments.

The clinical characteristics at presentation and the sites of involvement do not vary significantly with age (Table 2). Lymphomas are observed more frequently in elderly females, but this is probably related to the earlier mortality observed in males. Regardless of the age groups, localized NHL were more frequent in this study than it was previously reported: 19% for stage I disease and 20% for stage II disease. This may be due to the fact that such patients are less frequently included in prospective trials. Similarly, the type and frequency of sites of extranodal involvement did not change with age category (Table 2), the most frequent involved sites being bone marrow, spleen, gastrointestinal tract, head and neck, skin, and liver, as previously reported [17].

This study confirms the poor outcome observed in elderly patients with lymphoma. These elderly patients do not have specific histologic or clinical characteristics, nor they have more adverse prognostic features as shown in Tables 2 and 3. However, the outcome of elderly and young patients is clearly related to the number of adverse prognostic factors, as shown in Figure 4. Thus, age is not sufficient to discriminate patients with a poor outcome, but histologic classification and clinical staging are necessary to define a subgroup of patients with very poor outcome. As elderly patients do not have specific characteristics, treatment decisions must be defined according to histology and a clinical prognostic index as in all other age groups. This study was not designed to analyze the effect of the different therapeutic strategies used in the different centers, therefore, it is not possible to draw conclusions about the merits of the various treatment categories we have described (Table 4).

In summary, this implication would lead to the design of new studies addressing specifically patients older than 65 or 70 years wherein treatment protocols will be determined according to the histologic type and prognostic parameters specific for elderly patients such as PS, comorbid presence of other diseases, and the extent of the tumor.

*Study participants*

The pathologists and clinicians at each institution were, respectively, Wiq C. Chan and James O. Armitage (Omaha, NE), Randy Gascogne and Joseph Connors (Vancouver, Canada), Pauline Close and Peter Jacobs (Capetown, South Africa), Andrew Norton and T. Andrew Lister (London, UK), Ennio Pedroni and Franco Cavalli (Locarno, Switzerland), Françoise Berger and Bertrand Cöfler (Lyon, France), Faith Ho and Raymond Liang (Hong Kong), German Ott/Alfred Schauer and Wolfgang Hiddemann (Würzburg/Göttingen, Germany). The five visiting expert hematopathologists were Jacques Diebold (Paris, France), Kenneth A. MacLennan (Leeds, UK), H. Konrad Müller-Hermelink (Würzburg, Germany), Bharat N. Nathwani (Los Angeles, CA); and Dennis D. Weisenburger (Omaha, NE). James R. Anderson (Omaha, NE) and Pascal Roy (Lyon, France) provided statistical expertise regarding the study design and data analysis.

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11. The International Non-Hodgkin’s Lymphoma Prognostic Factors


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Original article

Epidemiology of the non-Hodgkin's lymphomas: Distributions of the major subtypes differ by geographic locations


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* See page 710 for list of participants

Summary

Background: There has been no previous systematic study of the distribution of the major subtypes of non-Hodgkin's lymphoma (NHL) across geographic regions, although there have been isolated reports of such differences.

Design: As part of a clinical evaluation of the International Lymphoma Study Group (ILSG) classification of NHL, we classified 1386 NHLs from eight different geographic sites (Omaha, NE, USA; Vancouver, BC, Canada; Cape Town, South Africa; London, England; Würzburg/Göttingen, Germany; Lyon, France; Locarno/Bellinzona, Switzerland; and Hong Kong) using the ILSG classification.

Results: Substantial differences were found in the distribution of the major subtypes of NHL across geographic regions ($P < 0.0001$). A greater percentage of follicular lymphoma was seen in North America, London and Cape Town (31% versus 14% at other sites). Peripheral T-cell lymphoma was more common in London, Cape Town and Hong Kong (9%) than elsewhere (3%). In Locarno/Bellinzona, higher percentages of mediatrastal large B-cell lymphoma (9% versus 2% elsewhere) and mantle cell lymphoma (14% versus 6% elsewhere) were seen. Angiocentric nasal T/NK-cell lymphoma was only seen in Hong Kong (8%) and Lyon (2%).

Conclusions: Our study provides evidence that the distribution of NHL subtypes differs by geographic region. These findings suggest that geographical differences in etiologic or host factors may be responsible for the observed differences in the distribution of cases across NHL subtypes.

Key words: epidemiology, geography, histologic subtypes, non-Hodgkin's lymphoma

Introduction

In 1994, the International Lymphoma Study Group (ILSG), a group of mostly European and American pathologists, proposed a new classification of lymphoid neoplasms [1]. Their approach was an attempt to define diseases which they thought could be recognized with contemporary morphologic, immunologic and genetic techniques. The proposed classification defined non-Hodgkin's lymphoma (NHL) subtypes using these techniques, but included no original data on patient characteristics and outcome, or the distribution of the various NHL subtypes.

Thus, in 1995, a group of hematopathologists, clinicians and statisticians were assembled to conduct a retrospective clinical evaluation of the ILSG classification of NHL [2]. The primary focus of this research effort was to evaluate the ability of experienced hematopathologists to apply the ILSG classification to NHL cases collected at various sites around the world and to describe the clinical characteristics and treatment outcomes for the common NHL subtypes. Because the cases came from eight countries on four continents, we were also able to compare the distributions of the major NHL subtypes across geographic regions.

Design

The specific details of the conduct of this study have been published elsewhere [2]. Briefly, nine institutions in eight countries were chosen to participate in the study, in part, because they were thought to have a case base representative of their geographical region. Up to 200 consecutive cases of previously-untreated NHL diagnosed between 1 January 1988 and 31 December 1990 were to be studied at each institution. A tissue biopsy other than bone marrow was required for the diagnosis of NHL. An panel of five review hematopathologists (see Appendix) traveled as a group over a period of eight months to each site and classified each case according to the ILSG classification [1]. In addition, a consensus diagnosis was obtained for each case. The consensus diagnosis was reached when at least four of the five review pathologists agreed upon the ILSG classification. For a diagnosis of follicular lymphoma or peripheral T-cell lymphoma, any grade or subtype was considered an agreement. For cases where consensus could not be reached, additional studies were performed and a consensus diagnosis was assigned based on an agreed upon algorithm.

The chi-square test for contingency tables was used to
Table 1. Distribution of the major non-Hodgkin’s lymphoma (NHL) subtypes by institution.

<table>
<thead>
<tr>
<th>Major NHL subtypes</th>
<th>Omaha (n = 200)</th>
<th>Vancouver (n = 200)</th>
<th>Capetown (n = 188)</th>
<th>London (n = 119)</th>
<th>Würzburg/Geitlingen (n = 203)</th>
<th>Lyon (n = 192)</th>
<th>Locarno/Bellinzona (n = 79)</th>
<th>Hong Kong (n = 197)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small B-lymphocytic</td>
<td>7%</td>
<td>1%</td>
<td>8%</td>
<td>8%</td>
<td>11%</td>
<td>8%</td>
<td>9%</td>
<td>3%</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>7%</td>
<td>7%</td>
<td>1%</td>
<td>7%</td>
<td>8%</td>
<td>7%</td>
<td>14%</td>
<td>3%</td>
</tr>
<tr>
<td>Follicular</td>
<td>32%</td>
<td>31%</td>
<td>33%</td>
<td>28%</td>
<td>18%</td>
<td>17%</td>
<td>11%</td>
<td>8%</td>
</tr>
<tr>
<td>Marginal zone B cell, MALT type</td>
<td>6%</td>
<td>7%</td>
<td>4%</td>
<td>5%</td>
<td>9%</td>
<td>13%</td>
<td>9%</td>
<td>10%</td>
</tr>
<tr>
<td>Diffuse large B cell</td>
<td>25%</td>
<td>25%</td>
<td>28%</td>
<td>27%</td>
<td>30%</td>
<td>25%</td>
<td>36%</td>
<td>56%</td>
</tr>
<tr>
<td>Primary mediastinal large B cell</td>
<td>0%</td>
<td>2%</td>
<td>3%</td>
<td>2%</td>
<td>0%</td>
<td>4%</td>
<td>9%</td>
<td>5%</td>
</tr>
<tr>
<td>Peripheral T cell</td>
<td>3%</td>
<td>3%</td>
<td>8%</td>
<td>8%</td>
<td>4%</td>
<td>4%</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>Anaplastic large T/null cell</td>
<td>2%</td>
<td>3%</td>
<td>3%</td>
<td>2%</td>
<td>1%</td>
<td>3%</td>
<td>0%</td>
<td>3%</td>
</tr>
<tr>
<td>Angiocentric nasal T/NK cell</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>2%</td>
<td>0%</td>
<td>5%</td>
</tr>
<tr>
<td>Other lymphoma diagnoses</td>
<td>15%</td>
<td>19%</td>
<td>12%</td>
<td>15%</td>
<td>19%</td>
<td>17%</td>
<td>10%</td>
<td>16%</td>
</tr>
</tbody>
</table>

Abbreviation: MALT = mucosa-associated lymphoid tissue.

assess evidence that the distributions of cases among histologic subtypes of NHL differed across geographic areas.

Results

The distribution of cases by histologic subtype for each geographic area is shown in Table 1. There was strong statistical evidence that the distribution of the cases among the major histologic subtypes of NHL differed substantially by geographic site (P < 0.0001). The distribution across NHL subtypes was similar for Omaha and Vancouver, except that fewer cases of small B-lymphocytic lymphoma were diagnosed in Vancouver. A greater percentage of follicular lymphoma was seen in North America, London and Capetown (28%-32%) than at the other sites (8%-18%). Peripheral T-cell lymphoma made up a larger percentage of the cases from London (8%), Cape Town (8%) and Hong Kong (10%) than from the other sites (1%-6%). Primary mediastinal large B-cell lymphoma accounted for 9% of NHL in Locarno/Bellinzona, whereas at other sites the percentages were 0%-4%. Mantle cell lymphoma was also more common in Locarno/Bellinzona (14%) than at the other sites (1%-8%). Angiocentric nasal T/NK-cell tumors were only seen in Hong Kong (8%) and Lyon (2%).

Discussion

Previous studies have reported considerable variation in the overall incidence of non-Hodgkin’s lymphoma (NHL) worldwide [3, 4]. Differences in the incidence of specific subtypes of NHL have also been reported, but these studies have been hampered by the use of multiple NHL classification systems. As part of a clinical evaluation of the International Lymphoma Study Group (ILSG) classification of NHL, five review hematopathologists reached a consensus diagnosis on 1378 cases of NHL reviewed over an eight month period in eight countries. As a result, we were able to compare the distribution of NHL cases among the common histologic subtypes in different geographic areas. It was not possible to calculate incidence rates because the sizes of the populations from which these cases arose were unknown. Note that the proportion of cases of a particular subtype at a site may be low (high) compared to other geographic sites either because the incidence of that subtype is low (high), or because the incidence is the same but the incidence of the other subtypes is higher (lower). Nevertheless, our findings suggest that some real differences in the incidence of certain subtypes of NHL exist among the geographic sites studied.

The distributions of cases studied in Omaha and Vancouver were similar. There were fewer cases of small lymphocytic lymphoma (SLL) seen in Vancouver, but this was thought to be due to a difference in practice patterns. Vancouver patients diagnosed with chronic lymphocytic leukemia (the leukemic counterpart of SLL) on the basis of a bone marrow biopsy typically would not have had additional biopsies of enlarged lymph nodes performed. Since cases were eligible for this study only if there was a tissue biopsy other than bone marrow available for the diagnosis of NHL, these patients would have been excluded from the study. Similar patients seen in Nebraska and elsewhere were more likely to have had biopsies of enlarged nodes, and would have been included with a diagnosis of SLL with marrow involvement.

Follicular lymphoma was seen more frequently in North America, London and Capetown (31% of all cases) than at other geographic sites (14%). Lower rates of follicular lymphoma have been reported in Asian populations [5] and developing countries [6]. The percentage of follicular lymphoma in Hong Kong (8%) was the lowest observed at any study site, and similar to that previously reported for Hong Kong [7]. In a study of the incidence of non-Hodgkin’s lymphoma in San Francisco/Oakland, California and western Washington state, Chinese-Americans and Japanese-Americans both had a reduced risk of follicular lymphoma, and the lowest risks were seen in those born in Asia [8]. The risk of diffuse lymphomas was similar in Chinese- and Japanese-Amer-
icans, and US-born whites. In a study of 1391 cases of NHL in patients age 15 years or older who were treated at six major medical centers in four representative areas of Thailand, follicular lymphoma constituted only 3.8% of all cases [9]. Follicular lymphoma also appears to make up only a small minority of cases of NHL seen in Oman (17%) [10], Pakistan (8%) [11], Nigeria (13%) [12] and Gabon (2%) [13]. The percentage of follicular lymphoma cases seen in mainland Europe (11%–18%) was also substantially less than that seen in North America, London and Capetown. The reasons for these differences is unknown, but may reflect a true decreased risk of follicular lymphomas among mainland Europeans.

Peripheral T-cell lymphoma represented a greater percentage of the cases in Cape Town (8%), London (8%) and Hong Kong (10%), as compared to the other sites (2%–6%). Indeed, including angiocentric nasal T/NK-cell lymphoma and anaplastic large T/null-cell lymphoma, T-cell lymphomas comprised 21% of the cases reviewed in Hong Kong. The percentage of T-cell lymphomas in Hong Kong Chinese has been previously reported to be 25% [14], and the percentage reported in Shanghai is similar (28%) [15]. The increased percentage of T-cell lymphomas observed in Hong Kong is not thought to be related to an increased incidence of T-cell lymphoma, but rather due to a lower incidence of follicular lymphoma [14, 16]. Angiocentric nasal T/NK-cell lymphoma was only seen in Hong Kong (8%) and Lyon (2%). This tumor is known to have a higher incidence in Asian countries and is associated with a high incidence of Epstein–Barr virus infection [17], as are some peripheral T-cell lymphomas seen in Asia.

Mantle cell lymphoma was more frequently seen in Locarno/Bellinzona, Switzerland (14%) than in the other sites (1%–8%). Few cases of mantle cell lymphoma were seen in Capetown (1%). Interestingly, the National Cancer Institute-sponsored Non-Hodgkin’s Lymphoma Pathologic Classification Project also found that mantle cell lymphoma (centrocytic type in the Kiel classification) was much more commonly seen in Milan, Italy (located some 70 miles south of Locarno) than in the American institutions which participated in the project [18].

One unexpected result of this study was the increased percentage of primary mediastinal large B-cell lymphoma seen in Locarno/Bellinzona, Switzerland. These tumors are indistinguishable from other diffuse large B-cell lymphomas histologically. Nevertheless, they appear to be different clinically, since these patients are younger and more often female than patients with diffuse large B-cell lymphoma [1, 2, 19]. Because of their unusual clinical presentation and their apparent increased percentage in Locarno/Bellinzona, they may have a unique pathogenesis.

In summary, we have shown that the distribution of NHL subtypes differs in different geographical areas around the world, and suggest that these differences may well reflect differences in etiologic factors or the host response to these factors in these geographic areas. Epidemiologic studies focused on specific subtypes of NHL in areas with an increased incidence are needed to elucidate such etiologic factors.

*Appendix

Non-Hodgkin’s Lymphoma Classification Project study participants

The pathologists and clinicians at each institution were, respectively, W. C. Chan, J. O. Armitage (Omaha, NE); R. Gascoyne, J. Connors (Vancouver, Canada); P. Close, P. Jacobs (Cape Town, South Africa); A. Morton, T. A. Lister (London, UK); E. Pedrazzini, F. Cavalli (Locarno/Bellinzona, Switzerland); F. Berger, B. Coiffier (Lyon, France); F. Ho, R. Liang (Hong Kong); G. Ott, A. Schurig, W. Hiddemann (Würzburg/Göttingen, Germany). The five review hematopathologists were J. Diebold (Paris, France); K. A. MacLennan (Leeds, UK); H. K. Müller-Hermelink (Würzburg, Germany); R. N. Nathwani (Los Angeles, CA); D. D. Weisenburger (Omaha, NE). N. L. Harris (Boston, MA) participated as a consultant regarding application of the International Lymphoma Study Group classification. J. R. Anderson (Omaha, NE) and P. Roy (Lyon, France) provided statistical expertise regarding the study design and data analysis.

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New Approach to Classifying Non-Hodgkin's Lymphomas:
Clinical Features of the Major Histologic Subtypes

By James O. Armitage and Dennis D. Weisenburger for the Non-Hodgkin's Lymphoma
Classification Project

Increasing knowledge about the biology of the non-
Hodgkin's lymphomas has led to new approaches in
classification. Rather than grouping lymphomas simply
based on cell size, cell shape, and growth pattern, it is
now possible to identify distinctive clinicopathologic
entities. In many cases, the existence of specific immuno-
logic and/or genetic features has confirmed the exist-
ence of these distinctive types of lymphoma. Since
patients will be given these diagnoses by pathologists,
it is important that clinicians be knowledgeable with
regard to their clinical characteristics. The findings for
the 13 most common lymphoma types that will be
encountered in clinical practice are presented here.

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NON-HODGKIN'S LYMPHOMAS are increasing in
incidence and will be diagnosed in more than 55,000
patients this year in the United States.1-2 These malignancies
have been increasing in incidence at a rapid pace, approximately 4%
year since 1950,3 and the mortality rate has been increasing in a parallel manner.

The classification of non-Hodgkin's lymphoma has been
modified several times in this century and has often been a
source of confusion and frustration for clinicians. Gali and
Mallory4 proposed the first widely used lymphoma classifi-
cation, but this classification was not useful clinically. In the
1950s, Rappaport et al.5 recognized the importance of
lymphoma-cell growth pattern and subdivided the non-
Hodgkin's lymphomas based on this characteristic, which
led to the clinically relevant classification that bears Rappa-
port's name. In the 1970s, it became apparent that non-
Hodgkin's lymphomas were tumors of the immune system
and were derived from T or B lymphocytes. This knowledge
led to the classifications of Lukes and Collins2 and Lennert
et al6,7 (Kiel classification). The Working Formulation was
proposed in 1982 in an attempt to unify the complex and
confusing lymphoma terminology and improve communica-
tion between pathologists and clinicians in different parts of
the world.8 The Working Formulation became the most
popular classification used in North America, whereas the
Kiel classification dominated clinical practice in Europe.

The 1980s and 1990s have been a time of rapid increase in
our knowledge of the biology of the immune system. New
insights into immunology and genetics have allowed the
recognition of a number of previously unrecognized types of
non-Hodgkin's lymphoma. In some cases, biologic observa-
tions confirmed the existence of entities that were suspected
on clinical and/or morphologic grounds. In 1994, the
International Lymphoma Study Group recognized the exist-
ence of these new entities and proposed a new classification
that has been referred to as the Revised European-American
Lymphoma (REAL) classification.9 These observations will
form the basis for a new World Health Organization classifi-
cation of lymphomas that will soon become available.

A recent retrospective study of the REAL classification
confirmed the clinical relevance of this approach.10 This
study was performed in eight countries throughout the world
and demonstrated that this new approach could be applied
more accurately than previous classification systems.10 The
data from that study form the basis of this report.

METHODS OF THE INTERNATIONAL LYMPHOMA
CLASSIFICATION PROJECT

The methods used have been published in detail10 and are
summarized here. Nine institutions in eight countries were
chosen to provide up to 200 consecutive cases of previously
untreated non-Hodgkin's lymphoma that were representa-
tive of the geographic region during the time between
January 1, 1988 and December 31, 1990. The first 200 cases
at each site that fulfilled the following criteria were selected
for the study. In all cases, tissue biopsy samples that were
adequate for diagnosis and classification were required and
all diagnostic pathology materials obtained before initial
therapy, including positive bone marrow specimens, were
included in the pathology review. Immunologic characterization as
to B- or T-cell origin, by whatever means in use at the institution,
was also required in all cases. Clinical data were also required in
all cases. The nine study sites provided a total of 1,403 cases.

At each institution, the pathology slides and reports for
each case were carefully reviewed by a designated site
CLINICAL FEATURES OF THE NON-HODGKIN'S LYMPHOMAS

pathologist. Five expert hematopathologists then traveled as a group to each of the nine sites to review and classify each case in each of the three major classifications. In each case, the expert was then presented with the immunophenotypic profile, along with any available cytogenetic and molecular genetic data, and the immunostains and/or flow cytometry report. After review, a second diagnosis was rendered in each classification. In addition to the independent diagnoses rendered by each of the expert pathologists, a consensus diagnosis was also reached in each case.

The International Prognostic Index was used to stratify patients within the various disease entities. Treatment outcome was measured using failure-free survival and overall survival. Failure-free survival was defined as the time from diagnosis to the first occurrence of progression, relapse after response, or death from any cause. Follow-up evaluation of patients who did not experience one of these events was censored at the date of last contact. Overall survival was measured from diagnosis to death from any cause, with surviving patient follow-up data censored at the last contact date. Estimates of failure-free survival and overall survival distribution were calculated using the method of Kaplan and Meier. Time-to-event distributions were compared using the log-rank test.

MAJOR LYMPHOMA SUBTYPES

The 13 most frequent clinical entities that are recognized in the new lymphoma classification are diffuse large B-cell lymphoma, follicular lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, peripheral T-cell lymphoma, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type, primary mediastinal large B-cell lymphoma, anaplastic large T/null-cell lymphoma, lymphoblastic lymphoma (T/B), Burkitt-like lymphoma, marginal zone B-cell lymphoma of nodal type, lymphoplasmacytic lymphoma, and Burkitt's lymphoma. These are the diagnoses that will be provided by pathologists to clinicians who care for patients with lymphoma for the ensuing years. Therefore, it is important that clinicians be acquainted with the clinical characteristics of these lymphoma types. Summaries of their frequency, clinical characteristics, biologic characteristics, and clinical course are presented in the following tables for easy reference.

The lymphomas are presented in order of their frequency of occurrence. Composite lymphomas are excluded, resulting in a total of less than 100%. A previous study has shown that expert hematopathologists can diagnose most subtypes accurately (ie, > 85%) when adequate material, immunophenotype, and clinical information are available. The exceptions are Burkitt-like lymphoma, nodal marginal zone lymphoma, and lymphoplasmacytic lymphoma. In these subtypes, lack of clear definitions are probably at fault. Burkitt-like lymphoma is probably a mixture of Burkitt's lymphoma and a variant more closely related to diffuse large B-cell lymphoma, with the former more frequent in pediatric cases and the latter more likely in older adults.

In conclusion, it is extremely important that clinicians recognize that the correct diagnosis of each of these lymphoma entities is only one of many important pieces of information necessary for planning patient care. The clinical prognostic characteristics as identified in the International Prognostic Index are also vitally important, since the prognosis of any particular patient is related to the biology of the specific lymphoma type and the patient's clinical prognostic characteristics. Combining this information will facilitate an accurate estimate of the prognosis and makes possible the development of a rational treatment plan for an individual patient.

This progress is not the last change in the classification of lymphomas we will see. New insights into the biology of lymphoma will continue to elucidate new clinicopathologic entities. Certainly, the large group of diffuse large B-cell lymphomas will be subdivided into more specific entities. The future for clinical investigation in the non-Hodgkin's lymphoma is promising. Hopefully, new insights into the biology of this group of disorders will lead to improved therapies.

APPENDIX

Study Participants

The pathologists and clinicians at each institution were as follows: Wing C. Chan and James O. Armitage (Omaha, NE); Randy Gassoyne and Joseph Connors (Vancouver, Canada); Pauline Cloke and Peter Jacobs (Cape Town, South Africa); Andrew Norton and T. Andrew Lister (London, United Kingdom); Enrico Pedrini and Franco Cavalli (Locarno, Switzerland); Françoise Berger and Bertrand Caillier (Lyon, France); Faith Ho and Raymond Liang (Hong Kong); German Ott/Alfred Schauer and Wolfgang Hiddemann (Würzburg/Göttingen, Germany).

The five visiting expert hematopathologists were as follows: Jacques Diebold (Paris, France); Kenneth A. MacLeannan (Leeds, United Kingdom); H. Konrad Müller-Hermelin (Würzburg, Germany); Bharat N. Nathwani (Los Angeles, CA); and Dennis D. Weisenburger (Omaha, NE).

Nancy L. Harris (Boston, MA) participated as a consultant regarding application of the International Lymphoma Study Group classification. James R. Anderson (Omaha, NE) and Pascal Roy (Lyon, France) provided statistical expertise regarding the study design and data analysis.
DIFFUSE LARGE B-CELL LYMPHOMA

This is the most frequently occurring non-Hodgkin's lymphoma. It contains predominantly lymphomas classified as diffuse large cell, diffuse mixed cell, or immunoblastic in the Working Formulation and lymphomas classified as diffuse centroblastic, diffuse centroblastic/centrocytic, and immunoblastic in the Kiel classification. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, diffuse large B-cell lymphoma was diagnosed accurately 87% of the time. Immunophenotyping improved the accuracy of diagnosis by 14%. Diffuse large B-cell lymphoma is a chemotherapy-curable lymphoma.

---

**Frequency**

- Age: years (Median 64, Range 14-98)
- Male: 55%
- Stage:
  - I: 12%
  - II: 13%
  - III: 16%
  - IV: 13%
- B symptoms: 33%
- Elevated LDH: 53%
- Karnofsky score ≤ 70: 24%
- Tumor bulk, cm
  - ≤ 5: 76%
  - > 10: 30%
- Any extranodal site: 71%
- > 1 extranodal site: 29%
- Bone marrow involved: 16%
- GI tract involved: 18%
- IPI score:
  - 0/1: 35%
  - 2/3: 46%
  - 4/5: 19%
- Typical immunophenotype:
  - CD20⁺, CD3⁻
  - t(14;18)(q32;q21)
  - t(8;14)(q24;q32)
  - t(3;14)(q27;q32)
  - BCL-2
  - C-MYC
  - BCL-6

**Oncogenes frequently involved**

---

*Abbreviations: LDH, lactate dehydrogenase; GI, gastrointestinal; IPI, International Prognostic Index.*
FOLLICULAR LYMPHOMA

The combined group of follicular lymphoma makes up the second most frequent type of non-Hodgkin's lymphoma. These lymphomas are classified as follicular small cleaved cell, follicular mixed cell, and follicular large cell lymphoma in the Working Formulation and predominantly as follicular centroblastic/centrocytic or follicular centroblastic lymphoma in the Kiel classification. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, follicular lymphoma was diagnosed accurately 94% of the time. However, subtyping of follicular lymphomas was less accurate. Immunophenotyping improved the accuracy of diagnosis by only 1%. Although there was an overall 5-year survival rate of approximately 72%, patients with a high International Prognostic Index score had a poor survival.
SMALL LYMPHOCYTIC LYMPHOMA

Small lymphocytic lymphoma makes up 6% of all non-Hodgkin's lymphomas. Since this often is the tissue manifestation of chronic lymphocyte leukemia, if patients who present with leukemia and predominantly blood and bone marrow involvement are included, the actual incidence would be higher. It contains predominantly lymphomas classified as small lymphocytic in the Working Formulation, but is called chronic lymphocytic leukemia in the Kiel classification. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, small lymphocytic lymphoma was diagnosed accurately 87% of the time. Immunophenotyping added only 3% to the diagnostic accuracy.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Median</th>
<th>Range</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
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<td>21-91</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>41%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karnofsky score ≤ 70</td>
<td>11%</td>
<td></td>
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<td>Tumor bulk, cm</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>≥ 5</td>
<td>59%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 10</td>
<td>13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any extranodal site</td>
<td>80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 1 extranodal site</td>
<td>29%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>7%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gl tract involved</td>
<td>3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/1</td>
<td>23%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/3</td>
<td>64%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/5</td>
<td>13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Typical immunophenotype</td>
<td>CD20+, CD3-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD10-, CD5+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD23-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>del(13q), +12</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
</tbody>
</table>

Characteristic cytogenetics
Oncogenes involved
MANTLE CELL LYMPHOMA

Mantle cell lymphoma is among the most frequent of the newly recognized subtypes of non-Hodgkin’s lymphoma. In the Working Formulation, mantle cell lymphoma is classified as diffuse small cleaved cell lymphoma most frequently, but also as follicular small cleaved cell lymphoma, small lymphocytic lymphoma, diffuse large cell lymphoma, and lymphoblastic lymphoma. In the Kiel classification, this lymphoma is most frequently classified as centrocytic lymphoma or centrocytodendroblastoid lymphoma. In the Non-Hodgkin’s Lymphoma Classification Project using histology, immunophenotyping, and clinical information, mantle cell lymphoma was diagnosed accurately 87% of the time. Immunophenotyping added 10% to the accuracy of diagnosis. Patients with mantle cell lymphoma have a striking male predominance, usually advanced disease, and a poor overall and failure-free survival, which belies the good survival usually anticipated with small cell lymphomas.

<table>
<thead>
<tr>
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<th>6% (n = 83)</th>
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<tr>
<td>Median</td>
<td>37-82</td>
</tr>
<tr>
<td>Male</td>
<td>74%</td>
</tr>
<tr>
<td>Stage</td>
<td>10%</td>
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<td>II</td>
<td>3%</td>
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<tr>
<td>III</td>
<td>6%</td>
</tr>
<tr>
<td>IV</td>
<td>1%</td>
</tr>
<tr>
<td>V</td>
<td>9%</td>
</tr>
<tr>
<td>8 symptoms</td>
<td>31%</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>40%</td>
</tr>
<tr>
<td>Karnovsky score &gt; 70</td>
<td>21%</td>
</tr>
<tr>
<td>Tumor bulk, cm</td>
<td></td>
</tr>
<tr>
<td>≥ 5</td>
<td>69%</td>
</tr>
<tr>
<td>≥ 10</td>
<td>23%</td>
</tr>
<tr>
<td>Any extranodal site</td>
<td>81%</td>
</tr>
<tr>
<td>&gt; 1 extranodal site</td>
<td>51%</td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>66%</td>
</tr>
<tr>
<td>GI tract involved</td>
<td>9%</td>
</tr>
<tr>
<td>IPI score</td>
<td>0/1 23%</td>
</tr>
<tr>
<td>2/3</td>
<td>54%</td>
</tr>
<tr>
<td>4/5</td>
<td>23%</td>
</tr>
<tr>
<td>Typical immunophenotype</td>
<td>CD20⁺, CD79⁺, CD10⁺, CD5⁻, CD23⁻, PRAD1⁺, H11, H14, h13q32</td>
</tr>
<tr>
<td>Characteristic cytogenetics</td>
<td>BCL1-PRAD1</td>
</tr>
<tr>
<td>Oncogenes involved</td>
<td>BCL1-PRAD1</td>
</tr>
</tbody>
</table>
PERIPHERAL T-CELL LYMPHOMA

The subgroup of peripheral T-cell lymphoma not otherwise specified represents the largest group of T-cell lymphomas in the REAL classification. For this report, angiocentric nasal lymphomas and human T-lymphotropic virus type 1 (HTLV-1)--associated lymphomas were excluded, which makes the results typical for those seen in most western countries. Peripheral T-cell lymphoma includes lymphomas with a wide variety of histologic appearances. Tumors in this subgroup were classified as diffuse small cleaved cell, diffuse mixed cell, diffuse large cell, and immunoblastic in the Working Formulation. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, peripheral T-cell lymphoma was diagnosed accurately 86% of the time. However, this was only true when immunophenotyping was available. Immunophenotyping improved the accuracy of diagnosis by 45%. Peripheral T-cell lymphomas have one of the lowest overall and failure-free survival rates.
MARGINAL ZONE B-CELL LYMPHOMA, MALT TYPE

This is among the most frequent of the newly recognized subtypes of non-Hodgkin's lymphoma. In this report, only low-grade MALT lymphomas are included. In the Working Formulation, MALT lymphomas were most commonly diagnosed as small lymphocytic lymphoma or small lymphocytic lymphoma with plasmacytoid characteristics, although some were called diffuse small cleaved cell lymphoma. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, marginal zone lymphoma of the MALT type was diagnosed accurately 86% of the time. Immunophenotyping added only 2% to the accuracy of the diagnosis. MALT lymphomas have one of the highest survivals of any subtype and even patients with a high International Prognostic Index score have a 5-year overall survival rate of 40%.
PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA

Primary mediastinal large B-cell lymphoma represents a diffuse large B-cell lymphoma that cannot be distinguished histologically, but presents as a clinical syndrome due to the presence of a large mediastinal mass. This syndrome is clinically distinctive in that it occurs predominantly in younger patients and has a female predominance. In the International Non-Hodgkin’s Lymphoma Classification Project using histology, immunophenotyping, and clinical information, mediastinal large B-cell lymphoma was diagnosed accurately 85% of the time. Immunophenotyping added 7% to the accuracy of diagnosis. The clinical course of these patients differed little from that of other patients with diffuse large B-cell lymphoma, although mediastinal radiotherapy may play an important role in their treatment.
ANAPLASTIC LARGE T-/NULL-CELL LYMPHOMA

Anaplastic large T-/null-cell lymphoma represents the second most common T-cell lymphoma in the REAL classification. Patients with anaplastic large T-/null-cell lymphoma were often classified as having anaplastic carcinoma or an undifferentiated malignant neoplasm before staining for the CD30 antigen and discovery of the characteristic chromosomal translocation between chromosomes 2 and 5 led to its recognition as a distinctive non-Hodgkin’s lymphoma. In the REAL classification, only anaplastic large-cell lymphomas with T or null immunophenotype are included in this category. In the Non-Hodgkin’s Lymphoma Classification Project using histology, immunophenotyping, and clinical information, anaplastic large T-/null-cell lymphoma was diagnosed accurately 85% of the time. Immunophenotyping contributed 39% to the accuracy of diagnosis. Patients with this subtype of lymphoma have a young median age and a male predominance, and have the best overall and failure-free survival rates of any large cell lymphoma.

<table>
<thead>
<tr>
<th>Frequency</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>34</td>
</tr>
<tr>
<td>Median Range</td>
<td>10-100</td>
</tr>
<tr>
<td>Male</td>
<td>69%</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16%</td>
</tr>
<tr>
<td>IE</td>
<td>3%</td>
</tr>
<tr>
<td>II</td>
<td>22%</td>
</tr>
<tr>
<td>III</td>
<td>10%</td>
</tr>
<tr>
<td>IV</td>
<td>10%</td>
</tr>
<tr>
<td>Any extranodal site</td>
<td>39%</td>
</tr>
<tr>
<td>&gt; 1 extranodal site</td>
<td>53%</td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>45%</td>
</tr>
<tr>
<td>GI tract involved</td>
<td>26%</td>
</tr>
<tr>
<td>Tumor bulk, cm ≤ 5</td>
<td>76%</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>17%</td>
</tr>
<tr>
<td>Karnofsky score ≥ 70</td>
<td>59%</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>59%</td>
</tr>
<tr>
<td>IPI score 0/1</td>
<td>61%</td>
</tr>
<tr>
<td>2/3</td>
<td>18%</td>
</tr>
<tr>
<td>4/5</td>
<td>21%</td>
</tr>
<tr>
<td>Typical immunophenotype</td>
<td></td>
</tr>
<tr>
<td>CD20*, CD3*</td>
<td></td>
</tr>
<tr>
<td>CD30*, CD15</td>
<td></td>
</tr>
<tr>
<td>EMA*, ALK*</td>
<td></td>
</tr>
<tr>
<td>F(2;5)(p23;q35)</td>
<td></td>
</tr>
</tbody>
</table>

![Graph showing survival rates](image-url)
LYMPHOBLASTIC LYMPHOMA (T/B)

The lymphoblastic lymphomas make up a small proportion of all non-Hodgkin's lymphomas and are associated with a low median age, male predominance, and advanced stage. In the Non-Hodgkin's Lymphoma Classification Project using histology, immunophenotyping, and clinical information, lymphoblastic lymphomas were diagnosed accurately 89% of the time. Immunophenotyping contributed 35% to the accuracy of diagnosis. This is an aggressive lymphoma with low overall and failure-free survival rates.
BURKITT-LIKE LYMPHOMA

Burkitt-like lymphomas could not be diagnosed accurately in the Non-Hodgkin's Lymphoma Classification Project. Using histology, immunophenotyping, and clinical information, the diagnostic accuracy was only 53%. The problem was the lack of precise definitions to separate this group from the diffuse large B-cell category or true Burkitt's lymphoma. The median age and clinical characteristics of this subgroup were intermediate between patients with diffuse large B-cell lymphoma and Burkitt's lymphoma, but more closely approximated diffuse large B-cell lymphoma.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>2% (n = 29)</th>
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</thead>
<tbody>
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<td>Age, years</td>
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</tr>
<tr>
<td>Median</td>
<td>57</td>
</tr>
<tr>
<td>Range</td>
<td>20-92</td>
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<tr>
<td>Male</td>
<td>59%</td>
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<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>19%</td>
</tr>
<tr>
<td>II</td>
<td>7%</td>
</tr>
<tr>
<td>III</td>
<td>4%</td>
</tr>
<tr>
<td>IV</td>
<td>22%</td>
</tr>
<tr>
<td>V</td>
<td>7%</td>
</tr>
<tr>
<td>B symptoms</td>
<td>39%</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>61%</td>
</tr>
<tr>
<td>Karnofsky score ≤ 70</td>
<td>27%</td>
</tr>
<tr>
<td>Tumor bulk, cm</td>
<td></td>
</tr>
<tr>
<td>≤ 5</td>
<td>92%</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>42%</td>
</tr>
<tr>
<td>Any extranodal site</td>
<td>79%</td>
</tr>
<tr>
<td>&gt; 1 extranodal site</td>
<td>43%</td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>21%</td>
</tr>
<tr>
<td>GI tract involved</td>
<td>29%</td>
</tr>
<tr>
<td>IPI score</td>
<td></td>
</tr>
<tr>
<td>0/1</td>
<td>30%</td>
</tr>
<tr>
<td>2/3</td>
<td>48%</td>
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<tr>
<td>4/5</td>
<td>22%</td>
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<td>Typical immunophenotype</td>
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</tr>
<tr>
<td>CD20+, CD3-</td>
<td></td>
</tr>
<tr>
<td>CD10+, CD5-</td>
<td></td>
</tr>
<tr>
<td>Trk</td>
<td></td>
</tr>
<tr>
<td>Characteristic cytogenetics</td>
<td></td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td></td>
</tr>
<tr>
<td>t(8;14)(q24;q32)</td>
<td></td>
</tr>
<tr>
<td>Oncogenes involved</td>
<td></td>
</tr>
<tr>
<td>BCL2, C-MYC</td>
<td></td>
</tr>
</tbody>
</table>
MARGINAL ZONE B-CELL LYMPHOMA, NODAL TYPE

Marginal zone B-cell lymphoma of the nodal type is one of the new forms of non-Hodgkin's lymphoma not recognized in the Working Formulation. In the Working Formulation, these lymphomas were most commonly found in the small lymphocytic subcategory, but were also sometimes diagnosed as diffuse small cleaved cell lymphoma, small lymphocytic lymphoma with plasmacytoid characteristics, or diffuse mixed cell lymphoma. In the non-Hodgkin's Lymphoma Classification Project using histology, immunophenotype, and clinical information, marginal zone B-cell lymphoma of the nodal type was diagnosed accurately 63% of the time. Immunophenotyping added 8% to the accuracy of diagnosis. These patients had an overall and failure-free survival similar to small lymphocytic lymphoma. These lymphomas are often currently diagnosed as monocytoid B-cell lymphoma.
LYMPHOPLASMACYTIC LYMPHOMA

Lymphoplasmacytic lymphoma is an uncommon diagnosis in the REAL classification, and includes patients that might also be diagnosed with Waldenström’s macroglobulinemia. In the Non-Hodgkin’s Lymphoma Classification Project using histology, immunophenotyping, and clinical information, lymphoplasmacytic lymphoma was diagnosed accurately 56% of the time. Immunophenotyping contributed 3% to the diagnostic accuracy. The clinical characteristics of this lymphoma were similar to those of small lymphocytic lymphoma.

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<tr>
<td>Male</td>
<td>53%</td>
</tr>
<tr>
<td>Stage I</td>
<td>7%</td>
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<tr>
<td>II</td>
<td>0%</td>
</tr>
<tr>
<td>III</td>
<td>0%</td>
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<td>IV</td>
<td>0%</td>
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<td>V</td>
<td>13%</td>
</tr>
<tr>
<td>VI</td>
<td>7%</td>
</tr>
<tr>
<td>E symptoms</td>
<td>7%</td>
</tr>
<tr>
<td>Elevated LDH</td>
<td>13%</td>
</tr>
<tr>
<td>Karnofsky score ≤ 70</td>
<td>18%</td>
</tr>
<tr>
<td>Tumor bulk, cm ≥ 5</td>
<td>22%</td>
</tr>
<tr>
<td>≥ 10</td>
<td>50%</td>
</tr>
<tr>
<td>Any extranodal site</td>
<td>25%</td>
</tr>
<tr>
<td>&gt; 1 extranodal site</td>
<td>100%</td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>40%</td>
</tr>
<tr>
<td>GI tract involved</td>
<td>73%</td>
</tr>
<tr>
<td>IPI score 0/1</td>
<td>7%</td>
</tr>
<tr>
<td>2/3</td>
<td>50%</td>
</tr>
<tr>
<td>4/5</td>
<td>25%</td>
</tr>
<tr>
<td>Typical immunophenotype</td>
<td>CD20+, CD3-; CD10-, CD5-; CD23+, cytoplasmic Ig+</td>
</tr>
<tr>
<td>Characteristic cytogenetics</td>
<td>t(9;14)(p13;q32), del 11q23</td>
</tr>
<tr>
<td>Oncogenes involved</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Abbreviation: cytoplasmic immunoglobulin.
BURKITT'S LYMPHOMA

This is a rare lymphoma in a clinical series that includes predominantly adults. Although this is a highly aggressive and clinically distinctive lymphoma requiring unique treatment approaches, the overall and failure-free survival rates are similar to diffuse large B-cell lymphoma.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>&lt;1% (n = 10)</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td>Median 31</td>
</tr>
<tr>
<td>Range</td>
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<tr>
<td>Male</td>
<td>89%</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>25%</td>
</tr>
<tr>
<td>II</td>
<td>12%</td>
</tr>
<tr>
<td>III</td>
<td>13%</td>
</tr>
<tr>
<td>IV</td>
<td>12%</td>
</tr>
<tr>
<td>B symptoms</td>
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<tr>
<td>Elevated LDH</td>
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<td>Karnofsky score ≥ 70</td>
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<tr>
<td>Tumor bulk, cm</td>
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<tr>
<td>&lt; 5</td>
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<tr>
<td>≤ 10</td>
<td>22%</td>
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<tr>
<td>Any extranodal site</td>
<td>78%</td>
</tr>
<tr>
<td>≥ 1 extranodal site</td>
<td>56%</td>
</tr>
<tr>
<td>Bone marrow involved</td>
<td>33%</td>
</tr>
<tr>
<td>Gill tract involved</td>
<td>11%</td>
</tr>
<tr>
<td>IPI score</td>
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<tr>
<td>0/1</td>
<td>57%</td>
</tr>
<tr>
<td>2/3</td>
<td>29%</td>
</tr>
<tr>
<td>4/5</td>
<td>14%</td>
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<tr>
<td>Typical immunophenotype</td>
<td>CD20+ , CD3</td>
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<tr>
<td>Characteristic cytogenetics</td>
<td>8(14)(q32;q21)</td>
</tr>
<tr>
<td>Oncogenes involved</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-MYC</td>
</tr>
</tbody>
</table>

Survival (%)

Overall Survival (%)

Failure-free Survival (%)

Years

Years

Years

Years
REFERENCES

Bone Marrow Transplantation for Acute Leukemia:
A Preliminary Report From the International
Bone Marrow Transplant Registry

R. Champlin for the Advisory Committee of the International Bone Marrow Transplant Registry

Bone marrow transplantation is increasingly used for the treatment of acute myelogenous leukemia and acute lymphoblastic leukemia. Factors associated with remaining in remission and with survival were evaluated by univariate analysis in 1,413 patients who received transplants from related HLA-identical donors since 1978 from 105 reporting centers.

Acute Myelogenous Leukemia

Comprehensive data from 739 patients were analyzed. The actuarial probability of survival at 4 years was 45% ± 7% (95% confidence interval [CI]) for 447 patients who received transplants in first complete remission (CR), 27% ± 13% in 114 transplant recipients in second CR, and 21% ± 8% in 178 patients with more advanced disease (P < .0002) (Fig 1). The actuarial probability of remaining in remission at 4 years was 75% ± 12% v 41% ± 23% v 35% ± 15%, respectively (P < .0001).

For patients receiving transplants while in first remission, factors associated with a higher probability of survival were younger v older age (P < .004) and no or mild v moderate or severe acute graft-v-host disease (GVHD) (P < .0001). Survival following use of methotrexate or cyclosporine (Cs) as agents to prevent GVHD tended to be higher (P < .02) than the use of T cell depletion of donor bone marrow. The only factor associated with a higher probability of remaining in remission was French-American-British (FAB) subtype M5 v M1 to M4 (P < .006). The use of higher v lower dose rates of total-body irradiation (P < .05) and the use of

From the Advisory Committee of the International Bone Marrow Transplant Registry (IBMTR): R.P. Gale, University of California at Los Angeles [Chairman]; F.H. Bach, University of Minnesota, Minneapolis; A.V. Barrett, Westminster Hospital, London; D.J. van Bekkum, Radboud Institute, Nijmegen, Netherlands; J.C. Biggs, St Vincent’s Hospital, Sydney, Australia; R.G. Blume, City of Hope National Medical Center, Duarte, CA; M.M. Borin, Medical College of Wisconsin, Milwaukee; K.A. Dickie, M.D., Anderson Hospital and Tumor Institute, Houston; E. Gluckman, Hôpital Saint-Louis, Paris; J.M. Goldman, Royal Postgraduate Medical School, London; R.A. Good, All Children’s Hospital, St Petersburg, FL; J.R. Hersh, Cleveland Clinic; R. Hong, University of Wisconsin, Madison; J.H. Kersey, University of Minnesota, Minneapolis; H.-J. Kolb, Universität München, Munich; A.M. Marmont, Ospedale San Martino, Genoa, Italy; T. Masuoka, Center for Adult Diseases, Osaka, Japan; H.A. Messner, Ontario Cancer Institute, Toronto; R.J. O’Reilly, Memorial Sloan-Kettering Cancer Center, New York; R.L. Powles, Royal Marsden Hospital, London; A.A. Rimm, Medical College of Wisconsin, Milwaukee; O. Ringden, Huddinge University Hospital, Sweden; J.J. van Rood, University Hospital, Leiden, The Netherlands; C. Rozman, Barcelona University, Spain; B. Speck, Kantonsspital, Basel, Switzerland; R.S. Weiner, University of Florida, Gainesville; and F.E. Zwaan, University Hospital, Leiden, The Netherlands.

Supported by Public Health Service Grants CA 40053 from the National Cancer Institute, DHHS, and AM 37352 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases, DHHS, Contract N01-AI-62530 from the National Institute of Allergy and Infectious Diseases, DHHS, and grants from the William Randolph Hearst Foundation, RGK Foundation, Ambrose Monell Foundation, Elsa U. Pardee Foundation, Sandoz Inc, and the Swiss Cancer League.

This is the 44th report from the IBMTR.

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methotrexate vs Cs were of borderline significance.

The probability of relapse was significantly higher in 23 identical twins who received transplants in first CR in comparison with the 447 HLA-identical sibling transplant recipients (P < .003); their survival rates were comparable, however, due to a reduced incidence of transplant-related complications in the twins.

ACUTE LYMPHOBLASTIC LEUKEMIA

Six hundred seventy-four patients with acute lymphoblastic leukemia (ALL) treated with high-dose chemoradiotherapy and bone marrow transplantation from HLA-identical sibling donors were analyzed. The actuarial probability of survival at 4 years was 43% ± 14% for 181 transplant patients in first CR,

35% ± 8% for 257 recipients in second CR, and 21% ± 8% for 236 patients with more advanced disease (P < .0001) (Fig 2).

The data were analyzed to evaluate outcome following transplantation in first or second remission among patients with ALL with features associated with a high probability of relapse if treated with chemotherapy. The results as summarized in Table 1 demonstrate that transplantation in second remission results in a significantly lower probability of 3-year survival and a higher probability of relapse than transplantation in first remission. Furthermore, among patients transplanted in second remission, those with ALL presenting with high-risk prognostic features have a significantly lower 3-year probability of survival and a higher probability of relapse than patients presenting at diagnosis with standard-risk features.

Table 1. Actuarial Probability of Survival and Relapse for Patients with ALL Presenting With High- or Standard-Risk of Prognostic Factors at Diagnosis

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>n</th>
<th>Risk Factors at Diagnosis</th>
<th>3-Year Probability (95% CI)</th>
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<tr>
<td></td>
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<td>Survival</td>
<td>P</td>
</tr>
<tr>
<td>1st CR</td>
<td>166</td>
<td>High</td>
<td>44% ± 14%</td>
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<tr>
<td>2nd CR</td>
<td>152</td>
<td>High</td>
<td>22% ± 9%</td>
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<tr>
<td>3rd CR</td>
<td>105</td>
<td>Standard</td>
<td>50% ± 13%</td>
</tr>
</tbody>
</table>

*High-risk features for patients treated with chemotherapy include one or more of the following: age ≥ 16; WBC ≥ 100 x 10^9/L; CNS involvement; B phenotype; L3 FAB subtype; T14:11; T1; and T12:22.
ACKNOWLEDGMENT

I thank D’Ettia W. Koser, Sharon K. Gurgul, and Todd M. Stiefert for help with data analysis and Karen A. Wiikowski for typing the manuscript.

Institutions contributing patient data for this report were as follows: Academisch Ziekenhuis St Raphael, Leuven, Belgium; Alta Bates Hospital, Berkeley, CA; Beijing Medical University, China; Bishop Clarkson Memorial Hospital, Omaha; Case Western Reserve University Hospitals, Cleveland; Centre Hospitalier Regional de Nancy, France; Centro Medico Nacional Marques de Valdecilla, Santander, Spain; Charing Cross Hospital, London; Chaim Sheba Medical Center, Tel-Hashomer, Israel; Christian Albrechts University, Kiel, Germany; Children’s Hospital Medical Center, Oakland, CA; Children’s Hospital of Philadelphia; Children’s Hospital Research Foundation, Cincinnati; Christchurch Hospital, New Zealand; City of Hope National Medical Center, Duarte, CA; Ciudad Sanitaria, Valencia, Spain; Cleveland Clinic; Clinica Puerta de Hierro, Madrid; Daini Red Cross Hospital, Nagoya, Japan; Dr Daniel Den Hoed Cancer Center & Rotterdam Radio-Therapeutic Institute, Rotterdam, The Netherlands; Emory University School of Medicine, Atlanta; Glasgow Royal Infirmary, Scotland; Hahnemann University, Philadelphia; J. Hills Miller Health Center, Gainesville, FL; Hôpital Bellevue, Saint Etienne, France; Hôpital Edouard Herriot, Lyon, France; Hôpital Jean Bernard, Poitiers, France; Hôpital Jean-Minjoz, Besancon, France; Hôpital Saint Antoine, Paris; Hôpital Saint Louis, Paris; Hospital de la Princesa, Madrid; Huddinge Hospital, Sweden; Institut J. Pauli I. Calmettes, Marsailles, France; Institute of Medical and Veterinary Science, Adelaide, Australia; Kanazawa University School of Medicine, Kanazawa-shi, Japan; Karl Marx Universität, Leipzig, Germany; Katholieke Universiteit Nijmegen, Nijmegen, The Netherlands; Kantonsspital Zurich; Kinderklinik Zurich; King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia; Klinika za Unutrašnje bolести KBC-Rebro, Zagreb, Yugoslavia; L.R. Cowan Cancer Clinic, Murray, UT; Lanzhou General Hospital, Gan Su Province, China; Loyola University Medical Center, Maywood, IL; M.D. Anderson Hospital and Tumor Institute, Houston; Marshfield Clinic, Marshfield, WI; McMaster University, Hamilton, Canada; Medizinische Universitätsklinik, Vienna; Medizinische Klinik, Zurich; Medizinische Universitätsklinik Basel, Switzerland; Medizinische Universitätsklinik, Tubingen, Germany; Memorial Sloan-Kettering Cancer Center, New York; Methodist Hospital, Houston; Midwest Children’s Cancer Center, Milwaukee; National Institute of Haematology and Blood Transfusion, Budapest; Northwestern University, Chicago; Oklahoma Teaching Hospital, Oklahoma City; Ontario Cancer Institute, Toronto; Ospedale Riuniti di Pesaro, Italy; Ospedale San Martino, Genoa, Italy; Prince of Wales Children’s Hospital, Randwick, Australia; Queen Elizabeth Hospital, Birmingham, England; Queen Elizabeth Hospital, Woolwookie, Australia; Rigshospitalet, Copenhagen; Rikshospitalet, Oslo; Roswell Park Memorial Institute, Buffalo; Royal Free Hospital, London; Royal Hobart Hospital, Australia; Royal Infirmary of Edinburgh; Royal Marsden Hospital, London; Royal Perth Hospital, Western Australia; Royal Postgraduate Medical School, London; Royal Victoria Infirmary, Newcastle Upon Tyne, England; Rush Presbyterian St Luke’s Medical Center, Chicago; S. Orsola University Hospital, Bologna, Italy; St James University Hospital, Leeds, England; St Mary’s Hospital, Seoul, Korea; St Vincent’s Hospital, Sydney, Australia; Sommelsweis University, Budapest; Tom Baker Cancer Centre, Calgary, Canada; Tata Memorial Hospital, Bombay, India; Tokai University School of Medicine, Kanagawa, Japan; Turku University, Finland; Univerzita La Sapienza, Rome; Université D’Angers, France; Université de Bordeaux, Pessac, France; Universités Saint-Luc, Bruxelles, Belgium; Università Cattolica, Pescara, Italy; Universität Ulm, Ulm/Donau, Germany; Universität Klinik, Vienna; Universitäts-Kinderklinik, Munich; University of Alabama, Birmingham; University of Barcelona, Spain; University of California at Los Angeles Center for Health Sciences; University of Cape Town Medical School, South Africa; University of Helsinki; University Hospital, Leiden, The Netherlands; University of Kansas, Kansas City; University of Kentucky, Lexington; University of Minnesota, Minneapolis; University of Oklahoma, Oklahoma City; University of Tokyo; University of Wisconsin, Madison; US Air Force Medical Center, Lackland Air Force Base, Texas; Westmead Centre, Australia; and Westminster Hospital, London.

REFERENCES

Occasional Survey

FACTORS ASSOCIATED WITH INTERSTITIAL PNEUMONITIS AFTER BONE-MARROW TRANSPLANTATION FOR ACUTE LEUKAEMIA

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Summary
Data from 176 patients with acute leukaemia given allogeneic marrow transplants between Jan. 1, 1977, and Dec. 31, 1980, and reported to the International Bone Marrow Transplant Registry were analysed retrospectively for prognostic factors associated with the development of interstitial pneumonitis (IPn). The overall incidence of IPn was 20% (36/176), and the disease was fatal in 21 of the 36 cases (58%). Three of more than thirty prognostic factors studied seemed to be associated with a low risk of IPn—pretransplant total body irradiation at a dose-rate ≤5.7 Gy/min, when compared with higher dose-rates (p<0.001); post-transplant immunosuppression with cyclosporin-A, when compared with methotrexate (p<0.0003); and transplantation of cells from female donors into female recipients, when compared with female donors and male recipients (p<0.0005). The low dose-rate of total body irradiation and the use of cyclosporin-A were considered as independent variables, but were confounded to the extent that it was not possible to determine if one or both factors were associated with a decreased incidence of IPn.

INTRODUCTION
Interstitial pneumonitis (IPn) is a frequent complication of allogeneic marrow transplantation in patients with leukaemia, aplastic anaemia, and severe combined immunodeficiency disease (SCID).14 The disease is associated with a severe morbidity and is fatal in more than half of the cases. No effective treatment is known. This study was undertaken to investigate pretransplant and posttransplant factors which might have prognostic significance for the development of IPn.

METHODS
Comprehensive data were reported to the International Bone Marrow Transplant Registry (Registry) by 28 transplant teams throughout the world for 215 leukaemia patients who received marrow transplants between Jan. 1, 1977, and Dec. 31, 1980. Included in this analysis are data from 176 patients with acute lymphoblastic and acute non-lymphoblastic leukaemia who were conditioned for transplantation with high doses (28 Gy) of total body irradiation (TBI) and who received methotrexate (MTX) or cyclosporin-A (CsA) post-transplant to prevent or modify graft-versus-host disease (GVHD). At the time of transplantation 117 patients were in complete remission and 59 were in partial remission or relapse. Excluded were 14 patients with chronic myelogenous leukaemia, 15 patients who received grafts from genetically identical twins, 3 patients who were prepared for transplantation with chemotherapy but without TBI, and 7 who received both MTX and CsA, no treatment, or other treatments as prophylaxis against GVHD.

Data were analysed by the use of univariate and multivariate techniques to investigate prognostic factors which might be associated with IPn. Among the factors studied were age, sex of patient, sex of donor, sex-match of donor and recipient, disease status, number of remissions, number of pretransplant transfusions, presence or absence of infection at the time of transplant, pretransplant antibiotic decontamination, time of isolation, duration of isolation, AB, HLA-match, HLA mismatch, cell culture-matched, interval from diagnosis to transplantation, year of transplant, pretransplant administration of donor buffy coat, pretransplant chemoradiotherapy regimen, total dose of TBI, dose-rate of TBI, dose of marrow/kg body weight, number of posttransplant transfusions, severity of GVHD, and drug used to prevent or modify GVHD.

The diagnosis of IPn was based on clinical and radiological observations, supported in some centres by open lung biopsy. The high mortality rate often enabled the diagnosis to be confirmed at necropsy.

RESULTS
The overall incidence of IPn was 20% (36/176), and 58% (21/36) of the patients died from the disease. The median interval from transplant to onset of IPn was 75 days (range, 6–236 days). The actuarial incidence of IPn in the first year after transplantation was 25% as calculated by life-table analysis.

In univariate analyses, three prognostic factors were significantly associated with IPn—dose-rate of TBI, drug used for prophylaxis against GVHD, and sex-match donor and recipient. IPn occurred in 6% (4/69) of patients who received TBI at a dose-rate of 2.5–3.1 Gy/min. The incidence was 30% (32/107) in patients who received TBI at a dose-rate of 6–30 Gy/min (median 7.5 Gy/min; the difference is significant (p=0.0001). Although the incidence of IPn was clearly decreased at dose-rates of TBI below 5.8 Gy/min, there seemed to be a threshold at 6–6 Gy/min, after which the incidence did not increase with higher dose-rates (fig. 1). IPn developed in 7% (4/56) of patients treated with CsA and 27% (32/120) of patients given MTX (p=0.0028).

Although both the lower dose-rate of TBI and CsA seemed to be associated with a low incidence of IPn, it is uncertain whether both were involved. Patients treated with low dose-rate TBI generally also received CsA; patients treated with higher dose-rate TBI were also often treated with MTX. Thus, there was confounding (p=0.53, p<0.0001) between the variables of TBI dose-rate and drug used for prophylaxis of GVHD (table I). IPn occurred in 1 out of 31 female patients given marrow from female donors and in 44% (16/36) of male patients given marrow from female donors (p=0.0001) (table II).

Male and female patients given marrow from male donors had an intermediate incidence of IPn (17%, 19/109).
IMMUNOPHENOTYPIC CLASSIFICATION OF LYMPHOMA AND LYMPHOCYTIC Lymphoma — AN EXPERIENCE IN THE SOUTH-WESTERN AREA OF THE CAPE PROVINCE OF SOUTH AFRICA

PETER JACOBS

The University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Observatory, The Cape, South Africa.

Abstract—Adequate tumour material was obtained for phenotypic classification using a standard library of monoclonal antibodies from 81 previously untreated patients with acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL), or lymphocytic lymphoma (LL). Sixty-one individuals were adults and 20 were children of 14 yr or younger. Fifty-eight of the patients (72%) had acute lymphoblastic leukaemia and the remaining 23 (28%) had chronic lymphocytic leukaemia or lymphocytic lymphoma.

Considering only the patients with acute lymphoblastic leukaemia (n = 58) the median age was 19 yr (range 2–69 yr); 9% were black, 43% were coloured, 48% were white, and the distribution between adults (n = 38) and paediatric patients (n = 20) was comparable.

Complete remission rate in the adults was 58% and in the paediatric group 85%. For the total group (n = 58) median duration of survival was 59 weeks for common, 39 weeks for null, 63 weeks for T-ALL, and 13 weeks for B-ALL subtypes. In both the common and the null groups overall and disease-free survival was superior in the children. In contrast, no difference was evident in the T-ALL group, which was also notable for its high incidence in young coloured males.

The 15 patients with CLL and eight with LL were adults and all the cells were phenotypically of B lineage: in view of the small numbers no comments are possible about ethnic differences. A multi-centre collaborative study is needed to define the epidemiology of haematologic malignancy in South Africa, with emphasis on differences among ethnically distinct subpopulations.

INTRODUCTION

The natural history and response to treatment of the haematologic malignancies varies between different races. Scientific information about these neoplasms is strikingly deficient from Africa. This is unfortunate in view of the unique opportunities that exist for such epidemiological study. In part, this may reflect reluctance of patients to leave their families and travel long distances to attend specialised clinics where the diagnostic and therapeutic expertise is available, and in part to the formidable problems of maintaining records when follow-up is unreliable, particularly once any improvement in symptoms has occurred [1]. Superimposed on these difficulties may be local cultural influences, including those of traditional healer or medicine-man, low-level of medical information, and a generally accepting philosophy to symptoms and signs of disease.

Systematic protocol studies since 1972 have been used to explore the presentation and management of haematologic malignancies at our institution. In view of the unique composition of our patient population, data has been accumulated on ethnic differences during the course of studies on acute leukaemia [2–4], chronic granulocytic leukaemia [5], myeloma [6], chronic lymphocytic leukaemia and the indolent lymphomas [7], and diffuse large cell lymphoma [8]. In general terms, three observations occur consistently. Firstly, median age at presentation is between 10 and 15 yr lower in blacks than in whites with the coloured population being intermediate. Secondly, from a catchment area of between 3 and 4 million people, of whom 12% are black, 53% are coloured, 33% are white, and 2% are Indian or Asian, the attendance at the specialised haematology clinic is approximately 10% for black, 40% for coloured, and 50% for white. This small but significant deviation from the composition of the overall population is probably attributable to limited availability of specialised services for haematologic disorders. Thirdly, many of our patients present late in the course of their disease with high tumour bulk and suboptimal nutritional status: such fundamental differences make direct comparison of results from other centres having a more affluent patient load and earlier stage disease unreliable. These factors can only be con-
MATERIALS AND METHODS

The reference population

The Groote Schuur Hospital drains between 3 and 4 million people, of whom approximately 12% are black, 53% are coloured, 33% are white and 2% are Asian. The average annual admissions approximate 75,000 and outpatient visits close to 1 million; in general medical or surgical beds approximately 10% of patients are black, 63% are coloured and 27% are white. The Department of Haematology admits, on average, 360 patients and sees 7200 outpatients annually, of whom 10% are black, 40% are coloured [9], and 50% are white. This latter pattern has been constant over the past decade and the differences between it and both the overall population and that for patients on the general hospital services probably reflects a referral pattern which has developed around the special interest and provision of diagnostic and therapeutic services for patients with haematologic problems, including myeloma, lymphoma and leukaemia.

No reliable national or local figures are available for the overall incidence of the major categories of haematologic malignancy. An approximation can be derived from the population in the catchment or drainage area and the statistics for the hospital department for the period January 1980 through June 1984 with an average annual new patient accrual rate for patients over the age of 14 yr being 30 for non-Hodgkin's lymphoma, 12 for Hodgkin's disease, 14 for chronic lymphocytic leukaemia, 6 for chronic granulocytic leukaemia, 30 for non-lymphoblastic leukaemia and 13 for acute lymphoblastic leukaemia. Within each of these disease-types, the ratio of black to coloured and white patients was 9:43:48, with an additional 1% Asian patients, with these figures being relatively constant. Furthermore, within each of these ethnic groups, the male to female ratio was 3:4:1, 1:2:2:1, and 1:24:1. The predominance of black males may reflect local cultural practice where larger numbers of males make up the work force and there is generally greater reticence of the more conservative rural black woman to leave her family, even to attend regional hospitals.

Study population (n = 81)

Fifty-eight patients with previously untreated ALL, 15 with CLL, and eight with LL were phenotypically classified using a standardised library of monoclonal antibodies provided by the LRF Centre as part of the collaborative group study of leukaemia subtypes (see paper by Greaves et al., in this issue).

All patients were previously untreated and entered onto study with informed consent. Fifty-eight had ALL, 15 had CLL and five had miscellaneous lymphocytic lymphoma.

Patient evaluation

All patients had detailed history taken and underwent full physical examination. Complete blood counts were carried out on the Coulter Counter Model S Plus [10] and using May–Grünewald–Giemsa staining were classified as ALL on the basis of the French–American–British (FAB) Co-operative Group’s proposal [11–13]. The diagnosis of chronic lymphocytic leukaemia was based on established criteria [14] and staged according to the Rai recommendations [15]. Lymphocytic lymphoma was classified according to the recommendations of the International Working Formulation [16]. Biochemical profile was determined using established methodology [17]. In vitro bone marrow culture studies were performed on aspiration samples. Blasts were examined by sheep erythrocyte rosette formation [18] and by direct and indirect immunofluorescence using antibodies against terminal deoxynucleotidyl transferase (TdT) [19] and a standardised library of monoclonal antibodies, including demonstration of surface immunoglobulin [20, 21], Dr and cALL antigens [22, 23].

Patient exclusions

The 15 patients with CLL are excluded from further analysis but are reported elsewhere [7, 24, 25]. The eight patients with miscellaneous lymphocytic lymphomas are not suitable for further analysis because the numbers are too small.

Chemotherapy programme

The adults were managed with conventional doses of vincristine, prednisone, L-asparaginase and Adriamycin. Those achieving complete remission underwent craniospinal prophylaxis and entered a three-year maintenance programme of oral 6-mercaptopurine and methotrexate with monthly drug intensification. The children were stratified into low or high risk; the former were treated with a St Jude regimen [26] and the latter with a modified LSA-L2 protocol [27].

Statistical analyses

These were carried out using established methods [28, 29].

RESULTS (TABLE I)

Age

The median age of the patients with ALL (n = 58) was 19 yr (range 3–69). There were 38 adults with a median age of 24.5 yr (range 16–69); the corresponding figures for blacks were 24 yr (range 19–48), coloureds 20.5 yr (range 16–62), whites 28 yr (range 16–69), and Asians 37 yr (range 34–64). Twenty of the patients were 14 yr or
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<th>Phenotype</th>
<th>Numbers and age</th>
<th>Percent</th>
<th>Male to female</th>
<th>Complete remission (%)</th>
<th>Survival (weeks)</th>
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<td></td>
<td></td>
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<td>47.5</td>
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Comparison of patient population, complete response, and actuarially predicted survival by phenotype between adults and children aged 14 yr or younger. The small numbers make statistical analysis unreliable but children are seen to generally have higher response rates and better survival than adults. A notable exception is T-ALL where, despite higher response rates in paediatric patients, survival is comparable to that found in the adults. B-ALL is an uncommon group but patients do uniformly poorly.

Editors note: Several of these cases were re-classified by the reference centre (see Table 2 in the paper by Greaves et al.). In particular 5 of the 17 cases of Null-ALL were classified as ALL of uncertain subtype. Three additional cases were considered unclassifiable ALL subtypes.
younger with a median age of 6.5 yr (range 3-14); the corresponding figures for blacks were 11 yr (range 9-13), coloureds 6 yr (range 3-12), and whites 6.5 yr (range 4-14).

**Race**

The 58 patients with ALL were made up of five (8%) blacks, 22 (40%) coloured, 28 (48%) white and three (4%) Asians. When subdivided into adult and paediatric individuals this distribution was constant.

**Common acute lymphoblastic leukaemia (Table 2)**

The median age of these 17 patients was 27 years; two were black, two were coloured, 12 were white and there was one Asian. There were 12 adults having a median age of 37.5 yr; one was black, one was coloured, nine were white and there was one Asian. Five of the patients were children, having a median age of 5 yr; one was black, one was coloured and three were white. The male to female ratio was 12.5; for the adults it was 9:3 and for the children 3:2.

The complete response rate for adults was 50%, and median survival was 23 weeks; for the children complete remission rate 100% and median survival was 76 weeks.

**Null acute lymphoblastic leukaemia (Table 3)**

The median age of these 17 patients was 19 yr; one was black, five were coloured, nine were white and two were Asians. There were 10 adults having a median age of 28.5 yr; there were no black patients, three were coloured, five were white and two were Asian. Seven of the patients were children, having a median age of 9 yr; one was black, two were coloured and four were white. The male to female ratio was 6:11; for the adults it was 5:5 and for the children it was 1:6.

The complete response rate for adults was 70% and median survival was 30 weeks; for the children complete remission rate was 86% and median survival was 62 weeks.

**T-acute lymphoblastic leukaemia (Table 4)**

The median age of these 18 patients was 18 yr; one was black, 12 were coloured and five were white. There were 12 adults, having a median age of 22.5 yr; one was black, eight were coloured and three were white. Six of the patients were children, having a median age of 6.5 yr; there were no black patients, four were coloured and two were white. The male to female ratio was 14:4, for the adults it was 9:3 and for the children 5:1.

---

**Table 2. Common acute lymphoblastic leukaemia**

<table>
<thead>
<tr>
<th></th>
<th>Haemoglobin</th>
<th>White blood count</th>
<th>Blasts</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>10.0</td>
<td>7.8</td>
<td>33%</td>
<td>77</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(6.6-14.8)</td>
<td>(2.1-71.2)</td>
<td>(0-87%)</td>
<td>(&lt;10-394)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td>10.3</td>
<td>8.7</td>
<td>26%</td>
<td>80</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(8.4-14.8)</td>
<td>(2.1-71.2)</td>
<td>(0-87%)</td>
<td>(&lt;10-356)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Children</strong></td>
<td>9.1</td>
<td>7.0</td>
<td>68%</td>
<td>40</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(6.6-12.6)</td>
<td>(4.2-27.2)</td>
<td>(14-75%)</td>
<td>(11-294)</td>
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</tr>
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<tr>
<td><strong>Median</strong></td>
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<td>(4.7-6.9)</td>
<td>(27-68%)</td>
<td>(&lt;10-40)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
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</tr>
<tr>
<td><strong>Coloured</strong></td>
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<td>7.0</td>
<td>26%</td>
<td>117</td>
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<tr>
<td><strong>Median</strong></td>
<td>(8.4-12.9)</td>
<td>(2.7-7.8)</td>
<td>(11-33%)</td>
<td>(77-394)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<td><strong>White</strong></td>
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<td>10.8</td>
<td>45%</td>
<td>56.5</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(6.6-14.8)</td>
<td>(2.1-71.2)</td>
<td>(0-87%)</td>
<td>(11-356)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
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### Table 3. Null acute lymphoblastic leukaemia

<table>
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<th>Haemoglobin</th>
<th>White blood count</th>
<th>Blasts</th>
<th>Platelets</th>
</tr>
</thead>
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<tr>
<td><strong>Total</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>8.1</td>
<td>9.1</td>
<td>58%</td>
<td>88</td>
</tr>
<tr>
<td>Range</td>
<td>(4.2–12.9)</td>
<td>(1.8–290)</td>
<td>(0–97%)</td>
<td>(5–517)</td>
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<tr>
<td><strong>Adults</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>87</td>
<td>15.3</td>
<td>63%</td>
<td>88</td>
</tr>
<tr>
<td>Range</td>
<td>(5.2–12.9)</td>
<td>(1.8–127)</td>
<td>(0–95%)</td>
<td>(27–517)</td>
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<tr>
<td><strong>Children</strong></td>
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<td></td>
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<td>Median</td>
<td>7.4</td>
<td>6.5</td>
<td>43%</td>
<td>63</td>
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<tr>
<td>Range</td>
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<td>(0–97%)</td>
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<td><strong>Black</strong></td>
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<td>65%</td>
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<tr>
<td><strong>Coloured</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>10.7</td>
<td>12.3</td>
<td>19%</td>
<td>71</td>
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<tr>
<td>Range</td>
<td>(5.2–12.9)</td>
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<tr>
<td><strong>White</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Median</td>
<td>8.0</td>
<td>7.7</td>
<td>58%</td>
<td>92</td>
</tr>
<tr>
<td>Range</td>
<td>(4.2–11.6)</td>
<td>(4.2–290)</td>
<td>(0–97%)</td>
<td>(53–461)</td>
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</tbody>
</table>

The complete response rate for adults was 67% and median survival was 46 weeks; for the children complete remission rate was 100% and median survival was 47.5 weeks.

**B-acute lymphoblastic leukaemia** (Table 5)

The median age of these six patients was 19.5 yr; one was black, three were coloured and two were white. There were four adults, having a median age of 33.5 yr; one was black, two were coloured and one was white. Two of the patients were children, having a median age of 7.5 yr; one was coloured and one was white. The male to female ratio was 2:4; for the adults it was 2:2 and for the children it was 1:1.

The complete response rate for adults was 25% and median survival was 4 weeks; in the children there were no responders and median survival was 7.5 weeks.

**Survival by phenotype**

Pooling the data from adults and children (Fig. 1), median survival is 59 weeks for common ALL, 39 weeks for null ALL, 63 weeks for T-ALL, and 13 weeks for B-ALL. Disease-free survival is 56 weeks for common ALL, 20 weeks for null ALL, and 66 weeks for T-ALL.

**DISCUSSION**

Ethnicity is a powerful prognostic factor influencing the natural history and response to treatment of patients with haematologic malignancy. Thus, comparisons between blacks and whites in New York State with Reef Towns in the Southern Transvaal reveal differences, suggesting that disease expression may be strongly influenced by the social milieu [30]. In a period now exceeding a decade, striking consistent differences have emerged between black, coloured and white patients at our institution. Of these, age has been most clearly documented, with the median for black being between 10 and 15 yr lower than white patients with the same disease, and the coloured population generally occupying an intermediate position.

Furthermore, although response rates for standardised management protocols are not different for race groups matched for other variables, late presentation with high bulk disease, often with sub-optimal nutritional status, show a trend to more frequent occurrence among referred black patients, although similar features may be found in less sophisticated individuals from the coloured and white race groups. In addition, sex distri-
distribution is a less striking but clearly demonstrable variable and, as in chronic granulocytic leukaemia, cytogenetic differences also appear to exist [5], although clinical presentation, haematologic features, response to treatment and survival rates could not be correlated with this finding.

Conventional morphology is insufficiently sensitive to reliably separate immunobiologically distinct subtypes of lymphoid neoplasia within the French-American-British (FAB) classification [12]. Monoclonal antibodies were used for this purpose to prospectively study the influence of age and race on remission rate and survival using standardised protocols for treatment in patients stratified by phenotypic subtype.

In the patients with common and null ALL the phenotype was distributed similarly, both between the adult and the paediatric age groups and, furthermore, there was no significant variation between black, coloured and white patients. Two additional points require comment. Firstly, both complete remission rate and median duration of survival is already showing a trend in favour of patients expressing the common acute leukaemia antigen. Secondly, age is predictably emerging as a favourable prognostic factor for both these immunophenotypic subgroups.

T-ALL is a particularly interesting cohort. Thus, while the age distribution is comparable to the other immunologically defined subgroups, the value of immunophenotypic classification is reflected in an unexpected finding with a clear predominance in young coloured males. Unlike other series reporting a prominence of mediastinal tumour, this finding was unusual in these patients. The complete response rate to standard chemotherapy was also not significantly different from common and null ALL patients, with adults achieving this status in 67% of cases and the children in 100%. However, survival shows two important differences. Firstly, as generally expected, in the paediatric age group this is inferior to the common and null ALL variants; a finding consistent with reports that the T-cell phenotype is an unfavourable prognostic factor in this age group [31]. Secondly, and by way of contrast, adults survive better than any of the other subtypes. To pursue this latter difference in more detail, comparative analysis has been
Table 5. B-Acute lymphoblastic leukaemia

<table>
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<tr>
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<th>Haemoglobin</th>
<th>White blood count</th>
<th>Blasts</th>
<th>Platelets</th>
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<td>20.0</td>
<td>49.5%</td>
<td>27.3</td>
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<tr>
<td><strong>Median</strong></td>
<td>(6.3–12.9)</td>
<td>(4.6–98.2)</td>
<td>(2–70%)</td>
<td>(19–221)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>(6.3–12.9)</td>
<td>(4.6–98.2)</td>
<td>(2–70%)</td>
<td>(19–221)</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td>11.0</td>
<td>17.0</td>
<td>41%</td>
<td>21.5</td>
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<tr>
<td><strong>Median</strong></td>
<td>(8.5–12.9)</td>
<td>(4.6–98.2)</td>
<td>(2–70%)</td>
<td>(19–159)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>(6.3–10.4)</td>
<td>(22.1–22.8)</td>
<td>(19–59%)</td>
<td>(32–221)</td>
</tr>
<tr>
<td><strong>Children</strong></td>
<td>8.3</td>
<td>22.4</td>
<td>39%</td>
<td>126.5</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(6.3–10.4)</td>
<td>(22.1–22.8)</td>
<td>(19–59%)</td>
<td>(32–221)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>(6.3–10.4)</td>
<td>(22.1–22.8)</td>
<td>(19–59%)</td>
<td>(32–221)</td>
</tr>
<tr>
<td><strong>Black</strong></td>
<td>8.5</td>
<td>98.2</td>
<td>62%</td>
<td>19</td>
</tr>
<tr>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Range</strong></td>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Coloured</strong></td>
<td>10.4</td>
<td>18.0</td>
<td>40%</td>
<td>23</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(10–11.5)</td>
<td>(16–22.8)</td>
<td>(19–70%)</td>
<td>(20–221)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>(10–11.5)</td>
<td>(16–22.8)</td>
<td>(19–70%)</td>
<td>(20–221)</td>
</tr>
<tr>
<td><strong>White</strong></td>
<td>9.6</td>
<td>13.3</td>
<td>30.5%</td>
<td>95.5</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>(6.3–12.9)</td>
<td>(4.6–22.1)</td>
<td>(2–59%)</td>
<td>(32–159)</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>(6.3–12.9)</td>
<td>(4.6–22.1)</td>
<td>(2–59%)</td>
<td>(32–159)</td>
</tr>
</tbody>
</table>

Fig. 1. Acute lymphoblastic leukaemia. Actuarially predicted survival by phenotype for pooled data from adults (n = 38) combined with children aged 14 yr or younger (n = 20). The notable feature is similarity in the curves for patients with common and T-ALL; the major influence being that of an unusually high occurrence of T-ALL in a subgroup of young coloured males. It is also notable that no plateau is yet evident for the overall population. Separate analysis by age is precluded by small numbers of patients in each immunophenotypically distinct subclass.
undertaken against a control group of patients immunologically classified as common or null-ALL [32].

In this study the complete remission rates were 71% for the T-ALL and 75% for the common or null group while actuarially predicted median duration of survival is 49 and 39 weeks, respectively. While these differences may reflect geographical and racial factors, they may equally well be explained by the thymic phenotype becoming a less reliable marker of poor prognosis with increasing age, and this is likely to be the result of the more aggressive clinical course of all phenotypic subgroups of acute lymphoblastic leukaemia in the adult.

CONCLUSION

Three points are emphasized by this study. Firstly, apparently homogeneous tumour morphology in lymphoproliferative disorders can be misleading and immunologic techniques provide a suitable methodology to more reliably define phenotypically distinct sub-populations. Secondly, the unexpected high incidence of T-ALL in young coloured males is in keeping with other evidence that ethnicity can exert an important and distinctive influence on disease expression. Thirdly, irrespective of race, age emerges as a favourable prognostic factor. Finally, there remains a striking paucity of properly assembled scientific data from countries in Africa, where a unique opportunity exists to define the potential impact of ethnic differences upon the natural history of different disease categories. The importance of the latter information is becoming increasingly appreciated as the role of viral infections and oncogene activation in the pathogenesis of human tumours such as the T-cell leukaemia–lymphoma syndrome is clarified [33]. All these cogent reasons for centres having the necessary expertise and patient populations collaborate in studies to explore this relatively neglected but important facet of geographical haematopathology.

Acknowledgements—Supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the National Cancer Association and the Medical Research Council. Data on the paediatric patients were contributed by Dr Cyril Karabus, Dr Paddy Harlcy, Dr Arthur Bird and laboratory staff. Monoclonal antibodies were supplied by Dr M. F. Greaves from the Leukaemia Research Fund Centre. Appreciation is expressed to Gail MeLellan for marker studies, Lucille Wood and Karen Edwards for data collection and analysis, Jackie Davies for typing, and the Medical Superintendent of Groote Schuur Hospital for permission to publish.

REFERENCES


OCCASIONAL SURVEY

BONE-MARROW TRANSPLANTATION FOR ACUTE LYMPHOBLASTIC LEUKAEMIA

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Summary

105 patients with acute lymphoblastic leukaemia (ALL) who received bone-marrow transplants from HLA-identical siblings during first or second remission had an actuarial survival rate at 4 years of 43±12% and an actuarial relapse rate of 32±12%. 98 patients with more advanced disease had a significantly lower probability of survival (15±5%) and a significantly higher probability of relapse (67±14%). Among high-risk patients, those transplanted in first remission had a higher survival probability (55±22%) than those transplanted in second remission (41±15%). Relapse rates in the two groups were comparable (28±24% and 31±19% respectively). Standard-risk and high-risk patients transplanted in second remission had comparable relapse rates, but there was a trend towards higher survival probability in standard-risk patients. Thus long-term disease-free survival in ALL can be achieved with bone-marrow transplantation. It is not yet certain whether transplants in first remission will result in higher survival rates than transplants in second remission; relapse rates were similar in the two groups.

INTRODUCTION

During the past 30 years substantial progress has been made in the treatment of acute lymphoblastic leukaemia (ALL).1-4 Modern intensive chemotherapy can produce remissions in more than 90% of children and more than 70% of adults with ALL.5-7 Long-term disease-free survival is achieved in up to 70% of children with the common form of ALL, and many are cured.8-14 There is, however, a subset of children with ALL with "high-risk" features, such as a high white-blood-cell (WBC) count, particular T or B cell phenotypes, specific chromosome abnormalities, or central-nervous-system (CNS) involvement at the time of diagnosis, who fare less well on chemotherapy.15-17 Similarly, patients aged over 16-20 have a less favourable prognosis.18,19-23 Cure rates in high-risk children and adults with ALL were <30% in most series. Several trials of intensive multidrug chemotherapy have shown improved results in the high-risk group, with survival of up to 50% at 3 years. Nevertheless, treatment with currently available chemotherapy fails in a substantial proportion of both children and adults with ALL. Once a patient with ALL relapses while receiving maintenance chemotherapy, the prognosis is extremely poor, with 2-4 year disease-free survival rates below 10% in most series.24-25

Several centres have reported cure rates of 10-20% with bone-marrow transplantation in patients with advanced resistant leukaemia,26-27 resistant or recurrent leukaemia has been the major cause of treatment failure.28 Attempts to reduce the relapse rate with more intensive preconditioning such as high-dose fractionated total-body irradiation (TBI) have been unsuccessful.29 In an effort to reduce the relapse rate, transplants have been carried out at an earlier stage of the disease, such as in second or third remission.30-32 A few transplants have been done in first remission in patients with high-risk forms of ALL.33-35

In the present report we compare the results of bone-marrow transplantation in patients with ALL at various stages of the disease. In particular, we have investigated survival and recurrence data for patients who, at diagnosis, were thought to be at high risk of recurrent leukaemia if treated with chemotherapy alone and/or who were at high risk because of one or more relapses while receiving chemotherapy.

PATIENTS AND METHODS

239 ALL patients who received bone-marrow transplants between Jan 1, 1977, and Dec 31, 1981, were reported to the International Bone Marrow Transplant Registry (IBMTR). Data for 204 of these patients whose donors were HLA-A and HLA-B identical siblings and whose cells were mutually unresponsive in mixed-lymphocyte-culture (MLC) tests were analysed. 35 of the 239 patients failed to meet the study criteria and were excluded from further analysis. Reasons for exclusion were MLC incompatibility (8 patients), fetal donors (7 patients), identical-twin donors (6 patients), parental donors (4 patients), MLC technical failures (4 patients), unrelated donors (3 patients), MLC and HLA incompatibility (1 patient), HLA incompatibility (1 patient), and HL-A technical failure (1 patient). Each of the 37 transplant centres that provided data for this study certified that they reported their consecutive transplant patients.

The status of the leukaemia was determined immediately before transplantation. Chemotherapy and was based on the criteria of Cancer and Leukaemia Group B.36 Patients with 5% or fewer blasts in the bone-marrow and a normal haemogram, physical examination, and performance status were judged to be in complete remission. Patients with 5-25% blasts in the bone-marrow were considered to be in partial remission, and those with more than 25% blasts were considered to be in relapse.
Patients were considered to be at standard risk or high risk at diagnosis on the basis of a prospective assessment at each transplant centre. Reasons for assignment of 66 of the 106 transplants in first or second remission to the high-risk category included one or more of the following: (a) age <1 or > 18 years (n = 44); (b) white blood cells > 50 × 10⁹/l (n = 12); (c) T or B cell surface markers (n = 22); (d) CNS involvement (n = 4), and (e) specific chromosome abnormality (n = 4). Patients without these findings at diagnosis are referred to as standard risk in this report.

Engraftment of transplanted bone marrow was classified by previously reported criteria. 29 Graft-versus-host disease (GVHD) was regarded as mild, moderate, or severe according to the IBMAT criteria. 26 A GVHD score was given, according to severity and duration of dermatitis, liver-function abnormalities, diarrhoea, and other clinical variables. Inasmuch as there are no signs, symptoms, laboratory tests, or histopathological tests generally accepted as pathognomonic of GVHD, this scoring system is arbitrary. For analysis, GVHD was evaluated as a continuous variable on a four-point scale from none to severe.

An abnormal chest X-ray with diffuse interstitial pattern, severe arterial hypoxaemia, or both in the absence of evidence of bacterial infection or congestive heart failure were the criteria for the diagnosis of interstitial pneumonia. In many cases open-lung biopsy or necropsy confirmed the diagnosis.

The closing date for the analysis was Feb 28, 1983. The minimum follow-up time from transplant for surviving patients was 14 months. In univariate analyses, analysis (without the Yates and Bonferroni correction) was used to test for differences between percentages and Student's t test (without correction for multiple contrasts) for differences between groups when a continuous variable was studied. Actuarial survival and recurrence curves were prepared with the life-table method, 44 and differences between curves were tested with the Lee-Desu statistic. 44 Graphical representation of the curves was terminated when fewer than 3 patients were at risk. Multivariate analyses were carried out using the log-linear analysis 45 and the Cox regression models. All probability values cited, with the exception of the beta errors, are from multivariate analyses.

In this type of investigation, in which group sizes are relatively small, it is often useful to compute the beta error. 47 This computation gives the probability of being wrong when one starts that there is no significant difference between two groups. The beta errors in this study were calculated on the assumption that the observed difference between two groups was the true difference.

RESULTS

We analysed the results of bone-marrow transplantation from HLA-identical siblings in 204 patients with ALL transplanted at various points during the course of their disease, including first remission (20), second remission (65), third remission (22), fourth to seventh remission (8), partial remission (21), and relapse (47). From preliminary study the post-transplant survival and recurrence data, it was apparent that the patients could be divided into two major groups—106 patients transplanted in first or second remission and 98 transplanted with more advanced disease (referred to as > second remission). Within each group there were no significant differences in survival or relapse rates or other transplant-related outcomes.

The distribution and frequency of 15 disease-related and transplant-related variables are presented in Table 1; there were no significant differences between the two cohorts of patients. WBC counts > 50 × 10⁹/l were present at the time of diagnosis in 17% of the first and second remission groups and 19% in the > second remission group. 4 patients in the first and second remission group presented with CNS leukaemia, and 10 patients in the > second remission group had CNS involvement previously or at the time of transplant. Chromosomal abnormalities thought to be associated with a poor prognosis by the transplant teams, such as the Philadelphia chromosome and the (t;4;11) translocation, were reported in 11 patients (6 in the first and second remission group and 5 in the > second remission group).

Leukaemia markers were studied in 106 patients: 6 had B-cell markers, 22 had T-cell markers, 27 had the common ALL antigen (CALLA), and 45 had neither B nor T-cell markers but CALLA studies were not done. Thus, 34 of 106 (32%) patients had B or T cell markers and were thought by the various transplant centres to have an unfavourable immune profile; their distribution between the two transplant cohorts was similar. Newer immune analyses, such as cytoplasmic Ig staining or tests with anti-T mononuclear antibodies, 46 were done in relatively few patients; it is therefore likely that a higher proportion of patients had an immune profile associated with an unfavourable prognosis. The distribution of donor/recipient sex-matching, was also comparable in the two groups.

Almost all patients were conditioned for transplantation with cyclophosphamide and TBI. 85 patients received additional antileukaemic drugs including amascrine, 1-aspargine, 1,3-bis[2-chloroethyl]-1-nitrosourea, busulphan, cytarabine, daunorubicin, doxorubicin, methotrexate, piperazinedine, prednisone, teniposide, thioguanine, and vincristine. Single-dose TBI was given to 137 patients (67%), 65 received fractionated TBI (32%), and 2 were irradiated. There were no significant differences in drug or radiation doses or schedules between the two transplant groups.

In summary, when each of 23 disease-related or transplant-related prognostic variables was analysed, there was no significant difference between the 106 first/second-remission patients and the 98 > second-remission patients.

Among the 106 patients transplanted in first or second remission, the actuarial probability of surviving 4 years was 43% (95% confidence interval [CI], 31.5-55%). In contrast, among patients transplanted with more advanced disease, the actuarial probability of 4-year survival was 15% (95% CI, 6.2-24%). The difference was significant (p = 0.01) (figure, A).
Post-transplant relapse data for the two transplant groups are presented in the figure (B). The actuarial probability of relapse among patients transplanted in first or second remission was 32% (95% CI: 20–44%) at 4 years. Patients who were transplanted when their disease was more advanced had a significantly higher (p<0.001) probability of relapse (57%, 95% CI, 53–81%).

Data on the 106 patients transplanted in first or second remission were analyzed in more detail to determine factors related to transplant outcome. We examined two questions: (a) whether high-risk patients at diagnosis had different outcomes when transplanted in first versus second remission, and (b) whether risk factors at diagnosis affected the outcome of patients transplanted in second remission.

66 of the 106 patients in first or second remission were considered to be high risk at diagnosis. These included all 20 first-remission transplants and 46 of 86 second-remission transplants. Age (<1 or >18 years), WBC (≥50×10⁹/L) and/or T or B cell leukemia were the major reasons for designating them high-risk patients (56 of 66), and these features were equally distributed between the two groups. With the exception of interval from diagnosis to transplant (median for first remission 5–5 months, second remission 17–5 months), the two groups were comparable for the variables shown in Table 1. Survival and relapse data for the high-risk patients transplanted in first versus second remission are given in Table 1. Two-year survival was 55±22% for first-remission transplants, compared with 41±15% for transplants in second remission (p=0.38; p<0.83). Relapse rates were 28±24% and 31±19%, respectively.

86 patients were transplanted in second remission—40 with standard-risk and 46 with high-risk features at diagnosis. The two groups were comparable for the variables indicated in Table 1. Survival and relapse data are shown in Table 2. 2-year actuarial survival was 53±17% in 40 patients without high-risk features at diagnosis and 41±15% in patients who were at high risk at diagnosis (not significant; p=0.46, p<0.81). Relapse rates were similar—28±16% and 31±19%, respectively.

Comparison of transplant outcome in patients without high-risk features transplanted in first versus second remission was not possible, because all 20 patients transplanted in first remission were identified at having high-risk features at diagnosis.

In addition to recurrent leukemia, GVHD and interstitial pneumonia (IPN), either alone or together, were other major causes of treatment failure. Mild, moderate, or severe GVHD developed in 104 (59%) of the 189 recipients who survived at least 3 weeks with evidence of engraftment and caused death in 54 (18%). In multivariate analyses, GVHD was significantly (p<0.001) associated with survival; the more severe the GVHD, the lower the survival rate. IPN developed in 61 (30%) of the 204 patients and was fatal in 46 (23%); all cases of IPN appeared within the first week, and the actuarial incidence was 9%. The occurrence of IPN was associated with a significantly lower (p<0.001) probability of survival. Post-transplant infections were often associated with GVHD and/or IPN and developed in 70% of the 204 patients. Infection was a primary or contributory cause of death in 51 patients (25%). There were no significant differences in the incidence or severity of GVHD, IPN, or infection between patients transplanted in first or second remission and those transplanted at a later stage of the disease. Failure to obtain engraftment was not a problem, occurring in only 1 of 190 patients who survived at least 3 weeks.

DISCUSSION

These results show that it is possible to achieve long-term leukemia-free survival in a substantial proportion of individuals with ALL, including those who do not respond to conventional and experimental chemotherapy. The best results were observed in patients transplanted in first or second remission, the 4-year probability of survival being 43% (±12%) and that of relapse 32% (±12%). Even patients...
with advanced disease benefited from transplantation, the probability of leukaemia-free survival being 15% (±5%) 4-7 years after transplant; these results probably are superior to those achieved with alternative therapeutic modalities.\(^{11-15}\) We and others have found that earlier transplantation benefits patients with acute myelogenous leukaemia (AML). Transplantation in first remission was found to be associated with the lowest probability of leukaemia relapse and the highest probability of long-term disease-free survival. The results of the present study show that earlier transplantation in ALL produces the same favourable trend as in AML; patients transplanted in first or second remission had a significantly higher probability of survival and of remaining in remission than did patients transplanted with more advanced disease.

Among patients who were at high risk at diagnosis, those who received a transplant in first remission had a higher survival rate than those transplanted in second remission (55±22% vs 41±15%), but the difference was not significant. However, the possibility of a beta-type error was high, and more patients and longer follow-up will be required to determine if this trend will persist. We found no suggestion of a lower relapse rate between patients in first and second remission (28±24% vs 31±19%).

Several other factors should be considered in the analysis of these data and their implications. First, only a proportion of high-risk patients who relapse during or following treatment with chemotherapy will achieve a second remission;\(^{12-15}\) estimates range from 10 to 70% depending on the clinical circumstances and treatment regimen. Secondly, the survival of ALL patients after relapse is affected to a major extent by whether relapse occurred during or after completion of maintenance chemotherapy.\(^{36,57}\) Thirdly, factors that accurately predict risk of relapse at diagnosis are of less (or no) consequence in patients who have remained in remission for at least 2 years.\(^{44}\) Thus the ultimate prognosis for high-risk patients who relapse will depend, in large measure, on whether they were on or off chemotherapy and the interval from remission to relapse; these factors are clearly interrelated as well. Finally, high-risk patients who achieve a second remission represent a biased population in that they have responded to chemotherapy. Because of the complexity of these and other issues, we urge caution in the interpretation of the aforementioned data on transplant outcome in high-risk ALL. The critical questions of the relative efficacy of transplantation in either first or second remission versus chemotherapy can only be answered in a prospective randomised trial.

We also analysed the results of transplantation in second remission in patients who had standard versus high-risk ALL at the time of initial diagnosis. Again a trend towards higher survival probability but no difference in relapse probability was observed. Results of transplantation in second remission were far more variable regardless of the initial risk assessment. Most of these 85 patients had relapsed while receiving maintenance chemotherapy. Even so, 2-year survival was 47±11% and the relapse rate only 29±12%. These results are at least as good as those reported with other therapeutic modalities in similar patients.\(^{13-15}\)

This study analysed the results of bone-marrow transplantation in patients with ALL at various stages. The results of transplantation in first or second remission were clearly superior to those achieved when the disease was more advanced. We were unable to determine whether transplantation in first remission conferred any survival advantage over transplants in second-remission patients who presented initially with high-risk features. Relapse rates did not appear to be affected. This study did not address the important issue of the relative efficacy of transplantation versus chemotherapy in the various stages of ALL. These questions need to be addressed by performing prospective randomised controlled clinical trials with appropriate stratification; some of these trials are in progress.

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REFERENCES


References continued on next page.
Marrow Transplantation for Acute Lymphoblastic Leukemia: Factors Affecting Relapse and Survival


Transplant outcome was analyzed in 690 recipients of bone marrow transplants (BMTs) for acute lymphoblastic leukemia (ALL) in first (n = 299) or second remission (n = 391). Actuarial 5-year leukemia-free survival was 42% ± 9% (95% confidence interval) and 26% ± 6%, respectively; relapse rates were 29% ± 9% and 52% ± 8%, respectively. Five-year leukemia-free survival was 56% ± 18% in children and 39% ± 10% in adults (P < .02) transplanted in first remission. In first-remission adults, non-T-cell phenotypes, bone-marrow donor-recipient mismatch and graft-vs-host disease (GVHD) were associated with decreased leukemia-free survival; inclusion of corticosteroids in the regimen to prevent GVHD was associated with increased leukemia-free survival. Variables associated with decreased leukemia-free survival after second-remission transplants were age ≥16 years and relapse occurring while on therapy. Variables associated with increased probability of relapse were similar for first- and second-remission transplants and included GVHD prophylaxis without methotrexate and absence of GVHD. In first-remission transplants, leukocyte count ≥50 × 10^9/L at diagnosis was also associated with increased relapse; in second remission, relapse while receiving chemotherapy was also associated with increased posttransplant relapse. These data emphasize the importance of both disease- and transplant-related variables in predicting outcome after BMT. They may be used to explain differences between studies, design future trials, and identify persons most likely to benefit from BMT.

C H E M O T H E R A P Y C U R E S a substantial proportion of patients with acute lymphoblastic leukemia (ALL). However, some patients have a high risk of relapse when treated with conventional regimens. These persons can be identified by certain patient- and disease-related variables. Treatment of high-risk patients has evolved over the past decade, and recent studies of intensive chemotherapy indicate improved results. Like high-risk patients, persons who relapse soon after achieving remission have a poor prognosis; few if any achieve long-term leukemia-free survival, although recent improvements in outcome are reported.

Bone marrow transplantation (BMT) has also been used to treat ALL. Results in first remission are encouraging. Transplants performed in second remission for persons who fail chemotherapy result in 5-year leukemia-free survival rates of 10% to 40% depending on the circumstances of relapse.

The relative roles of chemotherapy and BMT in high-risk children and adults with ALL and in persons who relapse after chemotherapy are controversial. Ideally, one would like to select patients for transplant whose predicted outcome with chemotherapy is poor. This approach is based on the assumption that factors which predict poor outcome with chemotherapy do not adversely influence transplant outcome. If, as suggested by several studies, similar disease and treatment characteristics are associated with poor prognosis after chemotherapy and BMT, the outlook for such patients may not be significantly improved by transplant. Deciding which is the better therapeutic option would thus be better addressed by comparing the results of chemotherapy and BMT in patients with similar prognostic features.

Prognostic factors for outcome of patients after BMT for ALL are currently ill-defined. Neither is there any agreement regarding the best transplant regimen. This study was undertaken to define patient and disease characteristics predictive of outcome after transplantation for ALL in first and second remission and to identify transplant-related approaches associated with decreased relapse and increased leukemia-free survival.
MATERIALS AND METHODS

Population

Patients with ALL (1,212) receiving allogeneic BMT between January 1, 1978 and December 31, 1986 were reported to the International Bone Marrow Transplant Registry (IBMTR) by 107 teams worldwide. Data for 690 recipients of HLA-identical sibling transplants were analyzed, including 299 patients transplanted in first complete remission (CR) and 391 patients transplanted in second CR. Excluded from this analysis were 177 patients who received marrow from a donor other than an HLA-identical sibling, 103 patients transplanted in third to eighth remission, 235 patients transplanted in relapse, and five patients whose disease status at the time of transplant was uncertain.

CR was defined as the absence of both medullary and extramedullary leukemia. Relapse includes both medullary and extramedullary sites. Patient, donor, and treatment characteristics are shown in Table 1. Age ranged from 1 to 48 years; 56 patients transplanted in first remission and 218 patients transplanted in second remission were aged <16 years. The median interval from diagnosis to first remission was 6 weeks. Among the 391 patients transplanted in second remission, 289 had their first relapse while receiving chemotherapy. The median duration of follow-up was 21 (range 4 to 93) months. Twenty-seven percent of patients were followed ≥ 3 years.

Children and adults transplanted in first remission differed significantly in race (58% white children v 77% white adults, P < .03), immune phenotype (54% T cell v 25% T cell, P < .001; 11% B cell v 5% B cell, P < .03), median leukocyte count at diagnosis (53 v 14 x 10^9/L, P < .0002), and presence of CNS leukemia at diagnosis (11% v 4%, P < .04) (Table 1). These differences in disease characteristics were presumably due to differences in selection criteria for transplant. Consequently, children and adults in first remission were analyzed separately. No significant differences in disease characteristics were evident between children and adults transplanted in second remission; they were analyzed as a single group.

Treatment regimens were similar for patients transplanted in first and second remission. Six hundred eighty patients (98%) received total body irradiation (TBI, median 10 Gy) in either single (42%) or multiple (58%) fractions. Eighty-five percent also received cyclophosphamide: 70% received cyclophosphamide alone, 9% received cyclophosphamide plus cytarabine, and 6% received cyclophosphamide plus other drugs. Fifteen percent received conditioning regimens that did not include cyclophosphamide: 12% received cytarabine with or without other drugs, and 3% received other drugs. Several methods were used to prevent graft-vs-host disease (GVHD). Most (97%) patients received one of the following: methotrexate with or without corticosteroids, cyclosporine with or without corticosteroids, methotrexate plus cyclosporine, or T-cell deletion of donor marrow with or without additional posttransplant immunosuppressive drugs (Table 1).

Statistical Methods

Actuarial probabilities of relapse and leukemia-free survival were calculated by standard life-table methods. Survival and relapse curves were terminated when fewer than three patients remained at risk (Figs. 1-4).

Identification of risk factors for relapse and survival was not attempted among children transplanted in first remission. They were heterogeneous both for disease characteristics and treatment regimens, and the number of patients was too small to allow adequate adjustment for this heterogeneity in multivariate analysis.

The variables in Table 1 were examined for their association with probability of relapse and leukemia-free survival for adults transplanted in first remission and all patients transplanted in second remission. The Lee-Desu statistic was used for univariate analysis. Variables significant in univariate analysis with P < .1 were examined in multivariate analysis using a proportional-hazards regression model with relapse and treatment failure as endpoint variables. Treatment failure was defined as either relapse or death from other causes.

GVHD was defined as moderate to severe (grade II through IV) acute GVHD and/or any grade of chronic GVHD for this analysis. GVHD was entered as a time-dependent covariate in the regression equation so that patients were scored as not having GVHD until the time of onset of grade II through IV acute or any grade of chronic GVHD.

Variables not significantly associated (P > .05) with probability of relapse and/or treatment failure were excluded from the regression model by a backward elimination procedure. Because of the multiple comparisons made, only P < .01 was considered significant. P between .01 and .05 was considered of borderline significance and should be interpreted with caution. Relative risks of relapse and treatment failure were derived from the proportional-hazards model and represent the risk for patients with unfavorable characteristics as compared with those with favorable characteristics, adjusted for effects of other variables in the model. Continuous variables were dichotomized to calculate relative risk. Results of multivariate analyses were examined for possible influence by differences among centers unaccounted for by recorded information by (a) repeating each analysis on the subgroup of patients transplanted in small centers and the subgroup transplanted in large centers; (b) entering center size (ie, the number of patients transplanted for ALL by a particular center during the study period) as a covariate in the regression equations and; (c) by repeating each analysis after excluding patients from each of the eight largest centers in turn. No substantial difference in the relative risks associated with the prognostic variables could be detected with any of these adjustments. Factors predictive of outcome were valid at both small and large centers. All P values are two-tailed and based on the results of multivariate analysis unless otherwise specified.

RESULTS

Results of transplants in first and second remission are shown in Table 2 and Fig. 1. Children aged <16 years transplanted in first remission had a 5-year probability of relapse of 27% ± 16% (95% confidence interval); their 5-year probability of leukemia-free survival was 56% ± 18%. Adults transplanted in first remission had a similar probability of relapse (30% ± 10%) but a lower probability of leukemia-free survival (39% ± 10%, univariate P < .02). Children and adults transplanted in second remission had a 5-year relapse probability of 52% ± 8%. The probability of leukemia-free survival was significantly lower for patients transplanted in second remission (26% ± 6%) than for children (univariate P < .0003) and adults (univariate P < .02) transplanted in first remission. Causes of treatment failure differed in these groups (Table 2). The most common cause of treatment failure among children transplanted in first remission was relapse. Adults transplanted in first remission had similar rates of relapse but increased mortality owing to transplant-related complications such as GVHD and interstitial pneumonitis. Children and adults transplanted in second remission had higher incidences of both relapse and fatal
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<td>Moderate</td>
<td>12 (23)</td>
<td>45 (19)</td>
</tr>
<tr>
<td>Moderately severe</td>
<td>2 (4)</td>
<td>25 (11)</td>
</tr>
<tr>
<td>Severe</td>
<td>3 (6)</td>
<td>26 (11)</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, total body irradiation; GVHD, graft-vs-host disease; CR, complete remission; NA, not applicable; CNS, central nervous system.

*Information available for 276 patients.

†Among patients surviving at least 21 days posttransplant with evidence of engraftment.

‡Among patients surviving at least 100 days posttransplant with evidence of engraftment.
Fig 1. Probability of relapse (A) and leukemia-free survival (B) after BMT for ALL according to disease status and age at time of transplant.

Fig 2. Probability of relapse after BMT of adults with ALL in first remission according to the method of GVHD prophylaxis used.

Fig 3. Probability of leukemia-free survival after BMT of adults with ALL in first remission according to whether or not steroids were administered in addition to methotrexate or cyclosporine to prevent GVHD.

Prognostic Factors for Adults in First Remission

Relapse. Use of methotrexate, especially when administered with corticosteroids, to prevent GVHD was associated with the lowest probability of relapse in this study (Table 3, Fig 2). Sixty-one adults who received methotrexate alone had a 3-year probability of relapse of 17% ± 14%; none of 34 first-remission adults who received both methotrexate and corticosteroids relapsed. In multivariate analysis, the proba-
(29% ± 20%, n = 27) or not receiving (35% ± 32%, n = 20) additional posttransplant immunosuppression. Only ten patients, too few for critical analysis, received both cyclosporine and methotrexate to prevent GVHD.

Development of acute and/or chronic GVHD was significantly associated with the probability of posttransplant relapse among adults transplanted in first remission. Patients without GVHD had a risk of relapse 3.1 times higher than patients with acute and/or chronic GVHD (P < .0004, group 2, Table 4). This effect was independent of the effect of methotrexate on relapse. Leukocyte level at diagnosis had a borderline association with relapse. Adults with leukocytes > 50 \times 10^9/L had a relative risk of relapse of 2.5 (P < .03) as compared with those in whom leukocytes were \leq 50 \times 10^9/L (group 2, Table 4). No other risk factor investigated, including age, time to achieve first remission, immune phenotype, and CNS leukemia at diagnosis, was significantly associated with the likelihood of relapse.

**Leukemia-free survival.** The highest probability of leukemia-free survival was observed in patients receiving corticosteroids for prophylaxis of GVHD in combination with either methotrexate or cyclosporine (Table 3, Fig. 3). The 3-year probability of leukemia-free survival was 67% ± 16% for 34 adults receiving methotrexate plus corticosteroids and 62% ± 27% for 20 receiving cyclosporine plus corticosteroids; it was 40% ± 13% for 61 patients who received methotrexate alone, 33% ± 16% for 69 who received cyclosporine alone, and 34% ± 20% for 47 who received T-cell-depleted BM. In multivariate analysis, patients receiving methotrexate or cyclosporine without corticosteroids had a significantly higher probability of treatment failure (death or relapse) than those who also received corticosteroids (relative risk 2.8, P < .004, group 1, Table 5). The reasons for treatment failure differed according to the prophylactic regimen used (Table 3). Interstitial pneumonitis was the primary cause of mortality among patients receiving methotrexate, whereas relapse was more common among patients receiving cyclosporine. Addition of corticosteroids to either methotrexate or cyclosporine was associated with a decreased incidence of both relapse and other fatal transplant complications. Patients who developed acute and/or chronic GVHD had a risk of treatment failure 1.9 times as high as those without GVHD (relative risk 1.9, P < .002, group 2, Table 5).

Donor-recipient sex match was significantly associated with leukemia-free survival. Females receiving grafts from male donors had a higher risk of treatment failure (relative risk 2.2, P < .002, group 3, Table 5) than those receiving grafts from females and than males receiving grafts from female or male donors. Female recipients of male transplants were similar to other patients in age, sex, immune phenotype, conditioning regimen received, and method of GVHD prophylaxis used. They did not have a significantly higher incidence of graft failure or GVHD. They did have somewhat higher leukocytes at diagnosis than other patients (median 35 × 10^9/L vs. 12 × 10^9/L, univariate P = .07). The reasons for treatment failure were not different for this group than for other sex-match combinations.

A borderline association was observed between immune phenotype and leukemia-free survival (group 4, Table 5). Patients with non-T-cell phenotypes (common ALL antigen [CALLA] null, or unclassified) had a higher risk of treatment failure than patients with T-cell ALL (relative risk 1.7, P < .02). The number of patients with B-cell ALL was too small for multivariate analysis. No other disease-related or treatment characteristic was significantly associated with leukemia-free survival among adults transplanted in first remission. In particular, the time to achieve CR, composition of the conditioning regimen, and posttransplant antileukemia treatment had no significant association with outcome.

**Table 2. Outcome and Reasons for Treatment Failure After BMT for ALL According to Disease Status and Age at Transplant**

<table>
<thead>
<tr>
<th>Factor</th>
<th>First CR, Age &lt; 16 yr</th>
<th>First CR, Age ≥ 16 yr</th>
<th>Second CR, All Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>56</td>
<td>243</td>
<td>391</td>
</tr>
<tr>
<td>Five-year probability of relapse (± 95% CI) (%)</td>
<td>27 ± 16</td>
<td>30 ± 10</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Five-year probability of leukemia-free survival (± 95% CI) (%)</td>
<td>56 ± 18</td>
<td>39 ± 10</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>Primary cause of treatment failure, n</td>
<td>9</td>
<td>37</td>
<td>114</td>
</tr>
<tr>
<td>Relapse</td>
<td>2</td>
<td>29</td>
<td>27</td>
</tr>
<tr>
<td>GVHD</td>
<td>0</td>
<td>13</td>
<td>29</td>
</tr>
<tr>
<td>IPn</td>
<td>1</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>Infection</td>
<td>4</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>126</td>
<td>254</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; IPn, interstitial pneumonitis.
Table 3. Outcome and Reasons for Treatment Failure After BMT for ALL Among Adults Transplanted in First Remission According to Method of GVHD Prophylaxis

<table>
<thead>
<tr>
<th>Factor</th>
<th>MTX Alone</th>
<th>MTX + Steroids</th>
<th>CSA Alone</th>
<th>CSA + Steroids</th>
<th>T-Cell Depletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>61</td>
<td>34</td>
<td>69</td>
<td>20</td>
<td>47</td>
</tr>
<tr>
<td>Three-year probability of relapse (± 95% CI (%))</td>
<td>17 ± 14</td>
<td>0</td>
<td>46 ± 24</td>
<td>27 ± 29</td>
<td>30 ± 17*</td>
</tr>
<tr>
<td>Three-year probability of leukemia-free survival (± 95% CI (%))</td>
<td>40 ± 13</td>
<td>67 ± 16</td>
<td>33 ± 16</td>
<td>62 ± 27</td>
<td>34 ± 20*</td>
</tr>
<tr>
<td>Primary cause of treatment failure, n</td>
<td>Relapse</td>
<td>5</td>
<td>0</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>GVHD</td>
<td>9</td>
<td>3</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GVHD + IPn</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IPn</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Infection</td>
<td>9</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>11</td>
<td>38</td>
<td>7</td>
<td>27</td>
</tr>
</tbody>
</table>

Abbreviations: MTX, methotrexate; CSA, cyclosporine.
*At 30 months.

Prognostic Factors for Patients Transplanted in Second Remission

Relapse. The regimen used to prevent GVHD was significantly associated with the probability of relapse among children and adults transplanted in second remission of ALL. Patients who received methotrexate with or without corticosteroids to prevent GVHD had a 3-year probability of relapse of 43% ± 11% (Fig 4). In contrast, the 3-year probability of relapse was 64% ± 15% for patients receiving cyclosporine and 68% ± 26% for patients receiving T-cell-depleted BM. Patients treated with cyclosporine or T-cell-depleted BM had a risk of relapse 3.1 times higher than that of methotrexate-treated patients (P < .0001, group 1, Table 4).

An antileukemia effect associated with GVHD was also observed. Patients without GVHD had a risk of relapse 2.0 times higher than those developing acute and/or chronic GVHD (P < .002, group 2, Table 4). This effect was independent of the effect of methotrexate on relapse.

Patients whose first relapse occurred while receiving chemotherapy had a risk of relapse posttransplant 3.3 times higher (P < .0002, group 4, Table 4) than that of patients whose first relapse occurred later. Information regarding the length of time patients were on or off maintenance chemotherapy was not available for analysis.

Leukemia-free survival. Age at time of transplant correlated with the probability of leukemia-free survival after second remission transplants (group 5, Table 5). Patients aged ≥16 years had a risk of treatment failure 1.6 times higher than that of younger patients (P < .0002). The time of first relapse was marginally associated with risk of treatment failure (group 6, Table 5): patients whose relapse occurred while they were receiving chemotherapy had a risk of treatment failure 1.7 times higher than that of patients whose relapse occurred after they completed chemotherapy (P < .02). No other pretransplant or treatment characteristic was significantly associated with probability of relapse or leukemia-free survival in multivariate analysis of patients transplanted in second remission.

DISCUSSION

Effect of GVHD Prophylaxis

This analysis demonstrates a significantly higher risk of relapse associated with use of cyclosporine and in vitro T-cell depletion of donor BM to prevent GVHD as compared with methotrexate in both first and second remission transplants for ALL. Similar but somewhat higher relapse rates have been reported by other investigators after use of methotrexate after BMT for adult ALL. An increased risk of relapse has been reported with T-cell-depleted transplants in both acute and chronic leukemia. T-Lymphocyte depletion has been postulated to increase leukemia relapse by removing cells responsible for a graft-versus-leukemia effect. This would not explain the relapse rates observed in recipients of non-

Table 4. Variables Associated With Relapse After BMT for ALL in Multivariate Analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Variable</th>
<th>Favorable</th>
<th>Unfavorable</th>
<th>First CR*</th>
<th>RR</th>
<th>P</th>
<th>Second CR†</th>
<th>RR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GVHD prophylaxis</td>
<td>MTX ± steroids</td>
<td>CSA or T-cell depletion</td>
<td>5.2</td>
<td>&lt;.0003</td>
<td>3.1</td>
<td>&lt;.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>GVHD</td>
<td>Acute and/or chronic</td>
<td>No acute or chronic</td>
<td>3.1</td>
<td>&lt;.0004</td>
<td>2.0</td>
<td>&lt;.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>WBCs at diagnosis</td>
<td>≤ 50 x 10⁹/L</td>
<td>≥ 50 x 10⁹/L</td>
<td>2.5</td>
<td>&lt;.03</td>
<td>NS</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Relapse occurred</td>
<td>Off chemotherapy</td>
<td>On chemotherapy</td>
<td>NA</td>
<td>NA</td>
<td>3.3</td>
<td>&lt;.0002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RR, relative risk; NA, not applicable.
*Adults only.
†Children and adults.
Table 5. Variables Associated With Treatment Failure After BMT for ALL in Multivariate Analysis

<table>
<thead>
<tr>
<th>Group</th>
<th>Variable</th>
<th>Favorable</th>
<th>Unfavorable</th>
<th>First CR*</th>
<th>RR</th>
<th>P</th>
<th>Second CR†</th>
<th>RR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MITX or CSA</td>
<td>With steroids</td>
<td>Without steroids</td>
<td>2.8</td>
<td></td>
<td>&lt;.004</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>GVHD</td>
<td>No acute or chronic</td>
<td>Acute and/or chronic</td>
<td>1.9</td>
<td></td>
<td>&lt;.002</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Sex match</td>
<td>M→M, F→F, F→M</td>
<td>M→F</td>
<td>2.2</td>
<td></td>
<td>&lt;.002</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Immune phenotype</td>
<td>T Cell</td>
<td>Not T cell</td>
<td>1.7</td>
<td></td>
<td>&lt;.002</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Patient age</td>
<td>&lt; 16 yr</td>
<td>≥16 yr</td>
<td>NA</td>
<td></td>
<td>NA</td>
<td>1.6</td>
<td></td>
<td>&lt;.0002</td>
</tr>
<tr>
<td>6</td>
<td>Relapse occurred</td>
<td>Off chemotherapy</td>
<td>On chemotherapy</td>
<td>NA</td>
<td></td>
<td></td>
<td>1.7</td>
<td></td>
<td>&lt;.02</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 4.
*Adults only.
†Children and adults.

T-cell-depleted transplants receiving cyclosporine unless cyclosporine inhibits the same graft-v-leukemia cells that are removed by T-cell depletion. Neither is the increase in relapse fully explained by an effect of cyclosporine on leukemia cells since T-cell depletion with and without cyclosporine was associated with similar probabilities of relapse. It is unlikely that methotrexate and steroids cause decreased relapse by increasing the incidence of GVHD and GVHD-associated antileukemia effects because the incidence of GVHD was similar in patients treated with methotrexate or cyclosporine. Furthermore, this effect persisted despite adjustment for the effect of GVHD in multivariate analysis. We believe that the association of methotrexate and corticosteroids with decreased relapse is most consistent with a direct antileukemia effect. Both drugs are active in ALL; they may work in the posttransplant period in a manner similar to maintenance chemotherapy. However, this contrasts with lack of a detectable effect of antileukemia maintenance therapy on outcome in this analysis and in a recent small single-center study. An effect of maintenance chemotherapy may be obscured by the effect of GVHD prophylaxis. Alternatively, differences in timing of therapy may be of crucial importance. Methotrexate and steroid prophylaxis for GVHD are administered immediately posttransplant, whereas maintenance chemotherapy is usually delayed for several weeks or more until stable engraftment occurs.

Methotrexate was associated with a fivefold decrease in relapse risk in first remission transplants and a threefold decrease in second remission transplants. No improvement in leukemia-free survival was observed in methotrexate-treated patients, however, due to increased mortality from interstitial pneumonitis. Corticosteroids appear to have a separate association with relapse (Table 3), reducing the risk of relapse by ~15% to 20% in both methotrexate- and cyclosporine-treated patients. This difference was not statistically significant. However, steroids used in addition to methotrexate or cyclosporine were associated with a 2.8-fold increase in leukemia-free survival in first-remission adult transplants due to modest decreases in both relapse and death from transplant-related complications. A similar effect was not observed in second-remission transplants. These findings suggest the desirability of randomized trials in ALL evaluating early use of corticosteroids for posttransplant maintenance therapy.

Effect of Other Transplant-Related Factors

GVHD. GVHD can have both favorable and unfavorable effects on transplant outcome. It may reduce the risk of leukemia relapse but increase transplant-related mortality. In first remission, the decreased relapse rate in patients with acute and/or chronic GVHD was more than offset by the increased risk of death from other causes. Consequently, patients with GVHD had lower leukemia-free survival (relative risk of treatment failure 1.9). In second remission, a similar decrease in relapse rate occurred but leukemia-free survival was not significantly altered. This may reflect the younger age of second-remission patients since the case fatality rate of GVHD is lower in younger persons.

Conditioning regimen. Leukemia relapse remains a major problem after BMT for ALL. To address this problem, addition of other drugs to cyclophosphamide and TBI and variations in radiation doses and schedules have been tried. Some studies used nodal irradiation or intrathecal chemotherapy to prevent relapse in sanctuary sites. Posttransplant leukemia maintenance therapy has also been administered. Because of the small number of patients available for analysis and the relative homogeneity of regimens used within single institutions, the impact of variation in these therapies can best be examined in multicenter studies. In this analysis of nearly 700 patients, none of the maneuvers decreased relapse rates. However, this study was limited to patients transplanted before 1987. Only small numbers of patients were treated with recently introduced regimens such as hyperfractionated TBI or combinations of high-dose etoposide or cytarabine and TBI, which are reported to affect outcome favorably.

Donor-recipient sex match. An unexplained finding was the high relapse rate and significantly increased risk of treatment failure in females receiving transplants from male donors. A similar observation was made in a recent European Bone Marrow Transplant Group analysis. No confounding prognostic variables could be detected, and there is no obvious explanation for the poor prognosis of such patients. One possibility is that host sensitization against H-Y antigens adversely affects the interaction between host and donor cells responsible for a putative graft-v-leukemia effect. However, the small number of patients available for study suggests that caution should be exercised until the
validity of this observation is confirmed or refuted with a larger sample.

Effect of Disease-Related Factors

The probability of leukemia-free survival with intensive chemotherapy overlaps results reported with transplants in first remission.\textsuperscript{11,12} It is not surprising, therefore, that controversy exists regarding their relative merits. Knowledge of whether prognostic variables for chemotherapy are similar to or different from those for transplantation would be useful in addressing this problem.

In this analysis, two prognostic factors reported to have an adverse effect on chemotherapy outcome\textsuperscript{5,12} were associated with adverse outcome of adults transplanted in first remission: leukocytes at diagnosis $>50 \times 10^3/L$ and non-T-cell phenotypes. Insufficient information was available to permit evaluation of the prognostic significance of null cell as compared with non-T, non-B, CALLA$^+$ ALL. These findings emphasize the need to stratify trials of chemotherapy $\rightarrow$ transplantation for cell type and leukocyte level at diagnosis. The interval between diagnosis and CR was not associated with transplant outcome in this study, in contrast to its importance in adults\textsuperscript{13,14} and children\textsuperscript{11,12} receiving chemotherapy, suggesting that patients requiring prolonged induction therapy might do better with transplantation. This should be confirmed in a randomized trial.

That no disease-related factor identifiable at diagnosis had prognostic value for patients transplanted in second remission suggests that the dominant prognostic feature in this group, the failure of primary therapy, overrides other variables. The situation at the time of first relapse was important: patients relapsing while still receiving chemotherapy had a 3.3-fold increase in risk of posttransplant relapse and a 1.7-fold increase in risk of treatment failure as compared with patients relapsing after discontinuing chemotherapy. The favorable prognosis of patients whose first relapse occurred off chemotherapy is consistent with similar observations in patients receiving chemotherapy,\textsuperscript{12,13} suggesting that prospective trials comparing chemotherapy $\rightarrow$ transplant in second remission should be stratified by this variable to allow valid comparison.

Transplantation of Children in First Remission

The data show that general policy has been to select for transplant only a small proportion of children with ALL in first remission and that these children have features perceived by the attending physicians as predicting a high risk of relapse if treated with chemotherapy.\textsuperscript{7} Thus, of the 56 children reported, most had T-cell ALL, CNS leukemia at presentation, or an initial leukocyte count $>50 \times 10^3/L$ (Table 1). Several had more than one adverse prognostic feature. Despite these poor prognostic features, leukemia-free survival was 56% at 5 years. Whether these results might have been achieved with chemotherapy is unknown; however, most recent studies report equivalent or better results. These data underline the need for prospective trials to compare transplantation in first remission with chemotherapy for children with high-risk ALL.

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REFERENCES


Factors Associated with Early Mortality Following Allogeneic Bone Marrow Transplantation for Acute Myelogenous Leukemia: A Report From the International Bone Marrow Transplant Registry

M. M. Bortin, R. P. Gale, and A. A. Rimm for the Advisory Committee of the Registry

Comprehensive data were reported to the International Bone Marrow Transplant Registry (IBMTR) by 28 bone marrow transplant centers regarding 156 patients with acute myelogenous leukemia (AML) who were treated with chemoradiotherapy and allogeneic bone marrow transplantation between 1978 and 1980. All patients reported to the IBMTR were included in the study. Sixty-one patients currently are alive and 95 have died; 68 (72%) of the deaths occurred in the first 6 months, 15 (16%) in the second 6 months, and 12 (13%) from 12 to 30 months posttransplant. The purpose of this study was to evaluate more than 20 pre- and posttransplant variables to determine whether they were associated with mortality within 6 months posttransplant or life-threatening complications during the first 6 months posttransplant. The major complications associated with 6-month mortality were infection, interstitial pneumonitis, and graft-versus-host disease (GVHD). Although recurrent leukemia was the primary cause of death for 63% (17/27) of the late deaths, it accounted for only 13% of the deaths within the first 6 months. Thus, recurrent leukemia was a major problem only for patients who survived more than 6 months posttransplant and will be the subject of a separate report from the IBMTR.

Pretransplant Clinical Findings

All 156 patients were included in the general designation of AML and all were considered to have a poor prognosis. The distribution of AML by subtypes was undifferentiated (12 patients), myelocytic (85 patients), promyeloctic (8 patients), myelomonocytic (35 patients), monocytic (8 patients), and erythroleukemia (8 patients). No significant differences were detected between the various subtypes of leukemia and outcome and, therefore, the data were pooled for statistical analysis. Bone marrow transplantation was performed in first complete remission (85 patients), second to fourth complete remission (26 patients), partial remission (22 patients) and relapse (23 patients). Patients were evenly distributed with respect to sex, and

The Advisory Committee of the Registry: Humphrey E. M. Kay, Royal Marsden Hospital, London (Chairman); Robert P. Gale, University of California at Los Angeles, (Vice-Chairman); Frits H. Bach, University of Minnesota, Minneapolis; Dirk W. van Bekkum, University of Rotterdam, Rotterdam; Morris M. Bortin, Mount Sinai Medical Center, Milwaukee; Karel A. Dicke, M.D. Anderson Hospital, Houston; Eliane Gluckman, Hôpital Saint Louis, Paris; Robert A. Good, Oklahoma Medical Research Foundation, Oklahoma City; Walter Hitzig, Kinderklinik, Zurich; Hans J. Koll, Institut für Hämatologie, Munich; Richard J. O'Reilly, Memorial Sloan-Kettering Cancer Center, New York; Alfred A. Rimmon, Medical College of Wisconsin, Milwaukee; Jon J. van Rood, Leiden University, Leiden; and Bruno Speck, Med Unniversitaetsklinik, Basel.

Supported in part by grants from the Burroughs-Wellcome Fund, Commission of the European Communities, Charles E. Culpeper Foundation, the Elizabeth Elser Doolittle Charitable Trust, Carl and Elisabeth Eberbach Foundation, March of Dimes Birth Defects Foundation, Ambrose Monell Foundation, Mount Sinai Medical Center (Milwaukee), Samuel Roberts Noble Foundation, Queen Wilhelmina Fund, Standos Limited, Swiss Cancer League, the Upjohn Company and Contracts N01-AI-02048 and N01-AI-22669 from the National Institute of Allergy and Infectious Diseases and the National Cancer Institute, USPHS.

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ranged in age from 1 to 62 years, median 24 years of age; 12% were 40 years of age or older at the time of transplantation. Donors and recipients were compatible at HLA-A, HLA-B, and in mixed leukocyte culture tests in 87% of the transplants. Donor-recipient histocompatibility was uncertain in 4% and incompatibility was present in 8%.

During the week pretransplant, all 156 patients were given high doses of antileukemic and immunosuppressive chemotherapy and, in addition, 154 patients received ≥ 8.0 Gy total body irradiation (TBI). Dose-rates of TBI ranged from 2.0 to 30.0 cGy/min with a median of 6.5. To prevent or modify graft-versus-host disease (GVHD), 100 patients were given methotrexate (MTX) and 51 received cyclosporin-A (CyA). Excluded from the analysis were 5 patients who received both MTX and CyA or neither.

STATISTICAL ANALYSIS

The prognostic factors that were included in the analyses are shown in Table 1. Multivariate techniques were used for data analysis because they provide an estimate of the effect of each prognostic variable on each outcome variable after adjusting for all other variables. The multiple logistic function analysis was used when the outcome variable could be measured on a binary scale and the Cox regression method was used to compare survival curves. The prognostic variables of dose-rate of TBI and drug used to prevent or modify GVHD were confounded and, as a consequence, it is not possible to state whether one or both of these variables were associated with outcome.

RESULTS

The 6-month survival rates for the 156 patients according to disease status at the time of transplantation were: first complete remission, 67% (57/85); second to fourth complete remission, 58% (15/26); partial remission, 45% (10/22); and relapse, 26% (6/23). There was a significant association between status of the disease at the time of transplantation and 6-month survival (p < 0.001). The main causes for treatment failure within the first 6 months posttransplant were infection (especially bacterial, fungal, and viral sepsis, and bacterial pneumonia), interstitial pneumonitis, and GVHD.

Among the 85 patients transplanted in first complete remission, the prognostic factors showing significant associations with 6-month mortality were the occurrence of moderate or severe GVHD and use of high dose-rates of TBI. Interstitial pneumonitis occurred with significantly increased frequency among patients given low doses of bone marrow cells, high-dose rate TBI, and/or MTX for prophylaxis of GVHD. Six-month outcome tended to be worse among older patients, those given many pretransplant transfusions, those who were infected at the time of transplantation, and if donors and recipients were incompatible in mixed leukocyte culture tests.

DISCUSSION

This study disclosed five prognostic factors that had significant (p < 0.01) associations with a favorable 6-month outcome: transplantation in first complete remission in comparison with more advanced disease status; administration of higher versus lower doses of bone marrow cells; occurrence of no or mild versus moderate or severe GVHD; use of lower versus higher dose-rates of TBI; and/or administration of CyA versus MTX as prophylaxis of GVHD. In addition, 6-month outcome tended to be favorable (0.10 <
Among younger in comparison with older patients, patient given few versus many pretransplant transfusions, absence versus presence of infection at the time of transplantation, and donor/recipient compatibility versus incompatibility in reciprocal mixed leukocyte culture tests.

Many of the prognostic factors found in this study that appeared to have an important impact on outcome can be controlled by the referring physician or by the bone marrow transplant team. If one assumes that at least some of these factors will be confirmed as having prognostic importance when tested under carefully controlled conditions, then one would expect improvement in the 6-month survival rates reported here. Further, as much as the mortality rate among patients who survived more than 6 months was relatively low, an increase in the 6-month survival rate may very well lead to an improved overall survival experience.

LIMITATIONS OF THIS REPORT

It is important to emphasize that the data summarized in this article were not based on a prospective randomized trial. Differences in practice, experience, and number of patients reported varied widely among the 28 centers that provided their data to the IBMTR. Thus, factors that appeared to have significant associations with outcome may have been influenced by the differences between centers. On the other hand, the random variability between centers tends to obscure significant differences and, we believe, the findings reported here represent conservative estimates of the associations that will be found when controlled clinical trials are employed.

CONCLUSIONS

Five prognostic factors were disclosed that had significant associations with outcome in the first 6 months following allogeneic bone marrow transplantation for AML. Four additional prognostic factors had suggestive associations with 6-month outcome. We recommend that controlled clinical trials be conducted to evaluate prospectively the influence on outcome of the prognostic factors reported here.
Graft-Versus-Leukemia Reactions After Bone Marrow Transplantation


To determine whether graft-versus-leukemia (GVL) reactions are important in preventing leukemia recurrence after bone marrow transplantation, we studied 2,254 persons receiving HLA-identical sibling bone marrow transplants for acute myelogenous leukemia (AML) in first remission, acute lymphoblastic leukemia (ALL) in first remission, and chronic myelogenous leukemia (CML) in first chronic phase. Four groups were investigated in detail: recipients of non-T-cell depleted allografts without graft-versus-host disease (GVHD), recipients of non-T-cell depleted allografts with GVHD, recipients of T-cell depleted allografts, and recipients of genetically identical twin transplants. Decreased relapse was observed in recipients of non-T-cell depleted allografts with acute (relative risk 0.68, \( P = .031 \)), chronic (relative risk 0.43, \( P = .011 \)), and both acute and chronic GVHD (relative risk 0.23, \( P = .0001 \)) as compared with recipients of non-T-cell depleted allografts without GVHD. These data support an antileukemia effect of GVHD. AML patients who received identical twin transplants had an increased probability of relapse (relative risk 2.58, \( P = .008 \)) compared with allograft recipients without GVHD. These data support an antileukemia effect of allogeneic grafts independent of GVHD. CML patients who received T-cell depleted transplants with or without GVHD had higher probabilities of relapse (relative risks 4.45 and 6.91, respectively, \( P = .0001 \)) than recipients of non-T-cell depleted allografts without GVHD. These data support an antileukemia effect independent of GVHD that is altered by T-cell depletion. These results explain the efficacy of allogeneic bone marrow transplantation in eradicating leukemia, provide evidence for a role of the immune system in controlling human cancers, and suggest future directions to improve leukemia therapy.

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In this study we examined the effects of bone marrow transplantation in 2,254 patients with early leukemia for evidence of graft-related antileukemia effects both in association with and independent of GVHD.

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MATERIALS AND METHODS

Population. Comprehensive data for 5,561 persons receiving bone marrow transplants for ALL, AML, or CML between January 1, 1978 and July 31, 1988, were recorded by 142 teams to the International Bone Marrow Transplant Registry (IBMTR). This study was restricted to the 2,254 patients with ALL in first remission (n = 439), AML in first remission (n = 1,046) or CML in chronic phase (n = 769) transplanted from an identical twin or HLA-identical sibling donor and receiving methotrexate, cyclophosphamide, and/or T-cell depleted bone marrow to prevent GVHD. Of these, 2,912 patients were alive at the time of transplant, 301 recipients of transplants from donors other than HLA-identical siblings, and 60 patients receiving either cyclophosphamide against GVHD (except for identical twins) or regimens other than methotrexate, cyclophosphamide, and/or T-cell depletion to prevent GVHD. Because the purpose of this study was to examine the influence of the graft upon leukemia relapse, 134 patients who failed to engraft or who did not survive long enough (2-21 days) to evaluate engraftment were also excluded from analysis.

Patient, disease, and treatment characteristics for the 2,254 subjects are shown in Table 1. Actuarial probabilities of relapse and leukemia-free survival at 5 years for patients with ALL were 30% ± 7% (95% confidence interval) and 44% ± 7%, respectively; for patients with AML, 23% ± 4% and 52% ± 6%, respectively; and for patients with CML, 23% ± 6% and 46% ± 5%, respectively. In 99% of cases, pretransplant conditioning consisted of total body radiation (median dose 10 Gy; range 5 to 16 Gy) and cyclophosphamide with (22%) or without (78%) other drugs. One hundred forty (6%) persons received total body radiation plus drugs other than cyclophosphamide. One hundred ten (5%) patients received chemotherapy alone, usually busulfan and cyclophosphamide. The distribution of conditioning regimens was similar for recipients of non-T-cell depleted and T-cell depleted transplants. Sixty-six of the 70 identical twin transplant recipients received total body radiation plus cyclophosphamide (n = 58) or other drugs (n = 8); three received busulfan and cyclophosphamide; one received busulfan and melphalan. Adjustment for type of conditioning regimen did not affect calculated risks of relapse or leukemia-free survival. Exclusion of patients not receiving total body radiation and cyclophosphamide also did not affect results. Results are presented with all patients included.

Five hundred ninety-one (27%) of 2,184 allograft recipients received posttransplant methotrexate with or without other drugs, excluding cyclophosphamide, to prevent GVHD; 825 (38%) received cyclophosphamide with or without other drugs, excluding methotrexate; 367 (16%) received cyclophosphamide plus methotrexate. Four hundred one (18%) received bone marrow depleted of T lymphocytes. Methods used for T-cell depletion included antibody with or without complement in 314 (78%) cases and physical techniques in the remaining cases. One hundred forty-two (35%) recipients of T-cell depleted bone marrow received no posttransplant GVHD drug prophylaxis; 215 (54%) received cyclophosphamide, and 44 (11%) received other drugs.

Acute GVHD was classified as absent (grade 0), mild (grade 1), moderate (grade II), moderately-severe (grade III), or severe (grade IV) using published criteria. Maximum overall severity of chronic GVHD was scored as absent, mild, moderate, or severe based on severity of skin and other organ involvement according to clinical judgement of the transplant team. In estimating the effect of GVHD severity in patients with both acute and chronic GVHD, patients with grade I acute GVHD and mild chronic GVHD were classified as having mild GVHD, those with grade II acute and moderate chronic as having moderate GVHD, and those with grade III to IV acute and severe chronic as having severe GVHD. One thousand three hundred forty-five (62%) allograft recipients developed grades I through IV acute GVHD. Seven hundred seven (38%) of 1,854 allograft recipients surviving with engraftment 100 days posttransplant developed chronic GVHD. In 551 (78%) persons, chronic GVHD appeared after acute GVHD. In 156 (22%), chronic GVHD developed without detectable antecedent acute GVHD (de novo chronic GVHD). Incidence and severity of acute and chronic GVHD in recipients of non-T-depleted and T-depleted grafts are shown in Table 2.

Complete remission was defined as absence of leukemia in the

<table>
<thead>
<tr>
<th>Table 1. Patient, Disease, and Treatment Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Median age, yr (range)</td>
</tr>
<tr>
<td>Male, n (%)</td>
</tr>
<tr>
<td>Median leukocyte count at D1, n x 10^3/L (range)</td>
</tr>
<tr>
<td>Median interval D1 to Tx, mo (range)</td>
</tr>
<tr>
<td>Total body radiation, n (%)</td>
</tr>
<tr>
<td>Median dose of TBI, Gy (range)</td>
</tr>
<tr>
<td>CY for conditioning, n (%)</td>
</tr>
<tr>
<td>Method of GvHD prophylaxis, n (%)</td>
</tr>
<tr>
<td>MTX alone</td>
</tr>
<tr>
<td>MTX + other</td>
</tr>
<tr>
<td>CSA alone</td>
</tr>
<tr>
<td>CSA + other</td>
</tr>
<tr>
<td>MTX + CSA</td>
</tr>
<tr>
<td>T depletion alone</td>
</tr>
<tr>
<td>T depletion + CSA</td>
</tr>
<tr>
<td>T depletion + other</td>
</tr>
<tr>
<td>None (Identical twins)</td>
</tr>
</tbody>
</table>

Abbreviations: D1 = diagnosis; Tx = transplant; TBI = total body radiation; CY = cyclophosphamide; MTX = methotrexate; CSA = cyclophosphamide.
Table 2. Severity of Acute and Chronic GVHD Among Recipients of Allogeneic Non-T-Cell Depleted and T-Cell Depleted Bone Marrow Transplants for Early Leukemia

<table>
<thead>
<tr>
<th>Variable</th>
<th>Non-T-Cell Depleted</th>
<th>T-Cell Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>1,783</td>
<td>401</td>
</tr>
<tr>
<td>Acute GVHD, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None (grade 0)</td>
<td>560 (31)</td>
<td>229 (57)</td>
</tr>
<tr>
<td>Mild (grade I)</td>
<td>471 (26)</td>
<td>88 (22)</td>
</tr>
<tr>
<td>Moderate (grade II)</td>
<td>400 (23)</td>
<td>50 (13)</td>
</tr>
<tr>
<td>Moderately-severe (grade III)</td>
<td>170 (10)</td>
<td>17 (4)</td>
</tr>
<tr>
<td>Severe (grade IV)</td>
<td>182 (11)</td>
<td>17 (4)</td>
</tr>
<tr>
<td>Chronic GVHD, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>904 (50)</td>
<td>243 (72)</td>
</tr>
<tr>
<td>Mild</td>
<td>290 (20)</td>
<td>59 (17)</td>
</tr>
<tr>
<td>Moderate</td>
<td>216 (14)</td>
<td>27 (8)</td>
</tr>
<tr>
<td>Severe</td>
<td>97 (6)</td>
<td>9 (3)</td>
</tr>
</tbody>
</table>

*Among patients who survived with engraftment ≥ 100 days posttransplant.

bone marrow and elsewhere. Leukemia relapse was based on hematologic criteria and/or postmortem studies. In patients with CML, relapse usually was accompanied by reappearance of the Ph1-chromosome. Reappearance of the Ph1-chromosome was not scored as relapse in the absence of hematologic or clinical evidence of leukemia since the biologic importance of such cytogenetic changes is unknown. Furthermore, the frequency with which recurrence of the Ph1-chromosome is recognized varies within and among centers because it depends on the frequency of cytogenetic examinations and the number of metaphases studied.

Statistical methods. Actuarial probabilities of relapse and leukemia-free survival were calculated using standard life table methods. Curves were terminated at 6 years or when fewer than five patients remained at risk.

To detect possible influences of allogeneic transplantation on leukemia relapse in association with and independent of GVHD, patients were categorized into a single reference and five comparison groups for multivariate analyses (Table 3). The reference group included recipients of non-T-cell depleted allografts without acute or chronic GVHD. Results in this reference group were compared with (1) recipients of non-T-cell depleted allografts with acute but not chronic GVHD; (2) recipients of non-T-cell depleted allografts with chronic but not acute GVHD; (3) recipients of non-T-cell depleted allografts with both acute and chronic GVHD; (4) recipients of transplants from genetically identical twin; and (5) recipients of T-cell depleted allografts with or without GVHD. Relative risks of relapse and treatment failure (defined as relapse or death from any cause) for each of the five comparison groups as compared with the reference group were calculated separately using Cox proportional hazards regression for all patients (in which the model was stratified by disease) and for patients with each disease. The risk of relapse for the reference group was assigned 1.00 in all multivariate analyses unless otherwise specified. Relative risks less than 1.00 indicate a risk of relapse less than the reference group and relative risks greater than 1.00 indicate a risk greater than the reference group.

To evaluate the effect of GVHD and posttransplant immunosuppression after T-cell depleted transplants and to compare overall risks of relapse between T-cell depleted and non-T-cell depleted transplants, different reference and comparison groups were used; these are specified in the text.

To avoid confounding of results by other variables associated with relapse and/or treatment failure, all regression equations were adjusted for variables associated (P < .05) with relapse and/or treatment failure in previous IBMTR analyses of patients with early leukemia: use of methotrexate, cyclosporine and/or corticosteroids to prevent GVHD, leukocyte levels at diagnosis, recipient age, organ impairment posttransplant, and donor–recipient sex match.

Analyses of GVHD. To accommodate changes in GVHD with time after transplant, patients were assigned to the three GVHD comparison groups described above and analyzed in a time-dependent fashion in the Cox regression models. All patients were considered to be in the “no GVHD” group at day 0. They were then assigned to the “acute GVHD only” group at the time of onset of acute GVHD, and their subsequent survival and relapse experience was compared with patients surviving a similar length of time without developing GVHD. Patients who later developed chronic GVHD were reclassified from the “acute GVHD only” group to the “both acute and chronic GVHD” group when chronic GVHD was diagnosed. Their subsequent survival and relapse experience was compared with patients surviving a similar length of time without developing GVHD. Patients developing chronic GVHD without prior acute GVHD were assigned to the “no GVHD” group until chronic GVHD developed; they were then assigned to the “chronic GVHD only” group. Their subsequent relapse and survival experience was compared with patients in the “no GVHD” group surviving a similar length of time.

P values are two-tailed and, unless otherwise specified, derived from multivariate analyses. Because a large number of statistical tests were performed, we consider only P values < .01 statistically significant and interpret values between .01 and .05 as indicating trends.

RESULTS

Recipients of non-T-cell depleted allografts not developing GVHD (reference group) had a 3-year probability of

Table 3. Unadjusted 3-Year Probability (±95% confidence interval) of Relapse After Bone Marrow Transplantation for Early Leukemia

<table>
<thead>
<tr>
<th>Study Group</th>
<th>ALL, First CR</th>
<th>AML, First CR</th>
<th>CML, CR</th>
<th>ALL Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Probability of Relapse (%)</td>
<td>N</td>
<td>Probability of Relapse (%)</td>
</tr>
<tr>
<td>All, Non-T-cell depleted</td>
<td>90</td>
<td>44 ± 17</td>
<td>228</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>Acute GVHD only</td>
<td>141</td>
<td>17 ± 9</td>
<td>330</td>
<td>27 ± 8</td>
</tr>
<tr>
<td>Chronic GVHD only</td>
<td>28</td>
<td>20 ± 19</td>
<td>64</td>
<td>22 ± 10</td>
</tr>
<tr>
<td>Acute and chronic GVHD</td>
<td>83</td>
<td>15 ± 10</td>
<td>132</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>12</td>
<td>41 ± 32</td>
<td>34</td>
<td>49 ± 21</td>
</tr>
<tr>
<td>All, T-cell depleted</td>
<td>64</td>
<td>34 ± 13</td>
<td>163</td>
<td>35 ± 12</td>
</tr>
</tbody>
</table>

Data are not adjusted for potential confounding variables that might influence relapse.

Abbreviations: CR, complete remission; CP, chronic phase.

*Reference group.
relapse of 25% ± 6% (95% confidence interval). Actuarial probabilities of relapse for the reference and comparison groups are shown in Fig 1 and Table 3. These values are not adjusted for possible confounding variables or for the time of onset of GVHD. Recipients with acute GVHD only, chronic GVHD only, or both had 3-year probabilities of relapse of 22% ± 5%, 10% ± 7%, and 7% ± 3%, respectively. Recipients of transplants from identical twins had a 3-year probability of relapse of 46% ± 15%. Recipients of T-cell depleted transplants had a 3-year probability of relapse of 41% ± 8%. Table 3 also shows the unadjusted 3-year probabilities of relapse for the reference and five comparison groups for each type of leukemia separately.

Relative risks of relapse derived from multivariate analyses are shown in Table 4. Patients who developed only acute GVHD had a relative risk of relapse of 0.68 (P = .01) compared with patients in the reference group; those with only chronic GVHD had a relative risk of 0.33 (P = .0001); and those with both acute and chronic GVHD had a relative risk of 0.23 (P = .03). The relative risks of relapse for recipients of identical twin and T-cell depleted transplants were 2.09 (P = .005) and 1.76 (P = .002), respectively.

Similarities and differences among the different leukemias were observed in the effect of GVHD. Identical twin transplants, and T-cell depletion on relapse (Tables 3 and 4). GVHD was associated with decreased relapse in all three types of leukemia; the risk was lowest in patients with acute and chronic GVHD. Acute GVHD only was associated with a decreased risk of relapse in ALL (relative risk 0.57, P = .004) but not in AML or CML (relative risks 0.78 and 1.15 respectively, P was not significant). A significant increase in relapse risk with identical twin transplants was observed only in AML (relative risk 2.68, P = .008). The increased risk of relapse associated with T-cell depleted grafts was significant only in CML (relative risk 5.14, P = .0001).

Severity of GVHD and leukemia relapse. To determine whether the antileukemia effect of GVHD was related to its severity, the risk of relapse associated with mild, moderate, and severe acute and chronic GVHD among patients receiving non-T-cell depleted allografts was compared with the risk of relapse in patients who did not develop GVHD. Risk correlated inversely with severity of GVHD (Fig 2). One hundred forty-one patients with mild GVHD had a relative risk of relapse of 0.50 (P = .02) or a twofold decrease in relapse risk as compared with those without GVHD; 72 patients with moderate GVHD had a relative risk of 0.22 (P = .009) or a 4.5-fold decrease in risk; none of 49 patients with severe acute and chronic GVHD relapsed (P = .04).

Effect of GVHD on relapse after T-cell depleted transplants. To determine whether T-cell depletion alters the antileukemia effect of GVHD, we compared the risk of relapse among recipients of T-cell depleted grafts with acute and/or chronic GVHD to the risk among recipients of T-cell depleted grafts without GVHD. Patients with GVHD had a risk of relapse 0.61 (P = .02) times that of patients without GVHD. The number of patients was too small to allow separate analysis of acute only, chronic only, and both acute and chronic GVHD groups.

Two analyses were performed to determine whether the increased risk of relapse associated with T-cell depletion could be accounted for by the decreased incidence and severity of GVHD.

First, the risk of relapse for recipients of T-cell depleted transplants without GVHD and those with acute and/or chronic GVHD was compared with the reference group of recipients of non-T-cell depleted allografts without GVHD (Tables 4). Recipients of T-cell depleted transplants without GVHD had a relative risk of relapse of 2.14 (P = .0001). This effect was significant only in CML (relative risk 6.91, P = .0001). Recipients of T-cell depleted transplants developing acute and/or chronic GVHD (relative risk 1.32, P = .25) had a risk of relapse not significantly higher than the reference group. However, when CML patients were analyzed separately, recipients of T-cell depleted transplants with GVHD had a higher risk of relapse than the reference group (relative risk 4.45, P = .003).

Second, the risk of relapse for T-cell depleted transplants was compared with the risk for non-T-cell depleted transplants using a proportional hazards model that adjusted for the incidence and severity of acute and chronic GVHD. After this adjustment the risk of relapse was 2.13 times higher (P = .0001) for T-cell depleted (n = 401) as compared with non-T-cell depleted (n = 1,783) grafts. No significant effect of posttransplant cyclosporine on relapse after T-cell depleted transplants was detected. Persons with CML receiving T-depleted grafts with and without cyclosporine (relative risks 5.37, P = .0001 and 4.74, P = .003, respectively) had significantly higher risks of relapse than the reference group.

Treatment failure. To determine whether altered risks of relapse affected the probability of leukemia-free survival, the risk of treatment failure (relapse or death from any cause) for patients in each comparison group was compared with the reference group (Table 5). Risk of treatment failure was significantly higher for patients with acute GVHD only (relative risk 1.84, P = .0001), with acute and chronic GVHD (relative risk 1.79, P = .0001), and recipients of T-cell depleted grafts (relative risk 1.59, P = .0003). Risks of treatment failure for patients with chronic GVHD only (relative risk 1.19, P = .45) and recipients of identical twin...
transplants (relative risk 1.07, P = .29) were not significantly different than the reference group. Among patients with acute and chronic GVHD, patients with mild GVHD had a risk of treatment failure 1.9 times lower (relative risk 0.53, P = .02) than the reference group, but those with moderate (relative risk 1.73, P = .04) or severe (relative risk 3.32, P = .0001) GVHD had risks higher than the reference group (Fig 2).

### Table 4. Relative Risk of Relapse After Bone Marrow Transplantation for Early Leukemia

<table>
<thead>
<tr>
<th>Study Group</th>
<th>ALL First CR</th>
<th>AML First CR</th>
<th>CML CP</th>
<th>All Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>RR</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Allogeneic, non-T-depleted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No GVHD</td>
<td>90</td>
<td>1.00</td>
<td></td>
<td>228</td>
</tr>
<tr>
<td>Acute GVHD only</td>
<td>141</td>
<td>0.36</td>
<td>.004</td>
<td>330</td>
</tr>
<tr>
<td>Chronic GVHD only</td>
<td>28</td>
<td>0.44</td>
<td>.16</td>
<td>54</td>
</tr>
<tr>
<td>Acute and chronic GVHD</td>
<td>84</td>
<td>0.38</td>
<td>.02</td>
<td>237</td>
</tr>
<tr>
<td>Syngeneic</td>
<td>12</td>
<td>0.99</td>
<td>.99</td>
<td>34</td>
</tr>
<tr>
<td>Allogeneic, T-depleted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Patients</td>
<td>84</td>
<td>1.20</td>
<td>.61</td>
<td>163</td>
</tr>
<tr>
<td>No GVHD</td>
<td>43</td>
<td>1.48</td>
<td>.33</td>
<td>83</td>
</tr>
<tr>
<td>Acute and/or chronic GVHD</td>
<td>41</td>
<td>0.98</td>
<td>.97</td>
<td>80</td>
</tr>
</tbody>
</table>

Relative risks are derived from multivariate Cox regression adjusting for leukocyte count at diagnosis, recipient age, organ impairment pretransplant, donor-recipient sex-match, and drug used to prevent GVHD. Abbreviations: RR, relative risk in comparison with reference group; CR, complete remission; CP, chronic phase. *Reference group.

### Discussion

Relapse rates differed for patients receiving non-T-cell depleted, T-cell depleted, and identical twin transplants. Because these groups received comparable pretransplant antileukemia therapy, these differences must arise for other reasons. The data presented suggest an additional antileukemia effect associated with bone marrow transplantation. This effect may have three distinct components: (1) antileukemia activity associated with clinically evident GVHD; (2) antileukemia activity independent of clinically evident GVHD; and (3) antileukemia activity independent of GVHD that is modified by T-cell depletion.

**Antileukemia effect of GVHD.** Prior studies of patients with advanced leukemia indicate a decreased risk of relapse associated with acute and/or chronic GVHD. In our study, relapse risk was also decreased in patients with early leukemia developing GVHD. The magnitude of this antileukemia effect correlated with GVHD severity; the lowest relapse rates were observed in patients with severe GVHD.

Assessing the relative effects of acute and chronic GVHD on transplant outcome is complex. Patients must survive sufficiently long to be at risk for acute GVHD and longer to be at risk for chronic GVHD. Consequently, individuals without GVHD include those dying before GVHD (and possibly leukemia relapse) can develop as well as those who survive without developing GVHD. The group with only acute GVHD includes patients with severe acute GVHD who do not survive sufficiently long to be at risk for chronic GVHD (or relapse), as well as those who survive without developing this complication. Additionally, individuals with acute GVHD are those most likely to develop chronic GVHD.

To accommodate changes in GVHD status over time and to determine which forms of GVHD were associated with an antileukemia effect, we used a statistical model that assigned patients to groups of acute GVHD only, chronic GVHD only.
or both in a time-dependent fashion. Using this model, we found decreased risks of relapse with both acute and chronic GVHD, although their relative importance differed for the three types of leukemia. Chronic GVHD had a stronger antileukemia effect in AML and CML, and acute GVHD had a stronger effect in ALL. Patients with both acute and chronic GVHD had the lowest risk of relapse.

There was an increased risk of nonleukemia deaths in patients with moderate to severe GVHD. Thus, despite its antileukemia effect, the presence of GVHD did not increase the likelihood of long-term disease-free survival, except for patients with mild acute and chronic GVHD.

**Allogeneic antileukemia effect.** The concept that allogeneic cells have an antileukemia effect independent of GVHD is supported by studies in mice, where T cells with GVL but not GVHD activity are identified. We found indirect evidence for such an effect in humans with AML. AML patients receiving allografts who did not develop GVHD had a lower risk of leukemia relapse than recipients of identical twin transplants. It may be that leukemia-associated antigens, not recognized by genetically identical immune cells, are recognized by allogeneic immune cells. Alternatively, the different relapse rate may reflect nonspecific effects of subclinical GVHD directed at minor histocompatibility antigens. We assume that the development of leukemia in twin transplant recipients represents relapse, but this was not formally proven. It is not possible to absolutely exclude increased susceptibility to leukemia or leukemia transformation in the immediate posttransplant period in genetically identical donor cells; however, this approach is supported by the fact that leukemia has not developed in any of the 70 twin donors. This GVL effect was greatest in AML, of borderline significance in CML, and absent in ALL. However, the number of identical twin transplants available for study in ALL and CML was small.

**Antileukemia effect of T cells.** The data in this study support an antileukemia effect of bone marrow transplantation for CML that is significantly altered by T-cell depletion. This effect is independent of the GVHD and GVL effects described above. It is presumably mediated by T cells but could result from some other cells or factors affected by T-cell depletion. T cells might interact with leukemia cells directly or by facilitating engraftment or producing lymphokines that affect growth of leukemia cells.

Part of the decreased antileukemia activity of T-cell depleted transplants is a consequence of decreased GVHD (Table 2). However, the fact that recipients of T-cell depleted transplants had an increased risk of relapse even after adjustment for GVHD suggests an additional antileukemia effect independent of GVHD. Among patients with CML, patients who developed GVHD after T-cell depleted transplants had a substantially higher risk of relapse than patients who received non–T-cell depleted grafts and did not develop GVHD.

In summary, these data provide evidence for antileukemia effects of bone marrow transplantation not explained by high-dose chemotherapy and irradiation. These activities may be mediated through several mechanisms. Advances in characterizing and controlling these effects are needed to improve results of bone marrow transplantation. It may also be possible to use these effects to treat leukemia outside the transplant setting.

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REFERENCES

Bone Marrow Transplantation for Acute Myelogenous Leukemia

Factors Associated With Early Mortality

Mortimer M. Borin, MD; Robert P. Gale, MD, PhD; Humphrey E. M. Kay, FRCPath; Alfred A. Rimm, PhD
for the Advisory Committee of the International Bone Marrow Transplant Registry

Comprehensive data were reported to the International Bone Marrow Transplant Registry, Milwaukee, regarding 156 patients with acute myelogenous leukemia who were treated with allogeneic bone marrow transplantation between 1978 and 1980. The minimum observation period was 15 months after transplant and most deaths occurred within the first six months. Prognostic factors were evaluated for associations with early mortality or life-threatening complications. Most early deaths were due to infections, interstitial pneumonitis, and graft-versus-host disease (GVHD). Multivariate analyses disclosed five factors with significant associations with early death or a major cause of early death: (1) disease status; (2) dose-rate of irradiation; (3) drug used to prevent GVHD; (4) severity of GVHD; and (5) dose of marrow cells. It is emphasized that several of the important prognostic factors are within the control of the referring physician or the transplant team.

(JAMA 1983;249:1166-1175)

IN THE 1970s, a number of transplant teams demonstrated that a small but significant proportion of patients with end-stage leukemia could be saved from certain death by treatment with high-dose chemotherapy followed by bone marrow transplantation. Analyses of data from these patients (whose leukemia was in relapse and refractory to traditional doses of chemotherapy) disclosed several important factors that were associated with success. The most important findings were that patients who underwent transplants relatively early in the course of the disease and while they were in good clinical condition had significantly higher survival rates than those who underwent transplants later in the disease and while they were in poor clinical condition. Largely on the strength of these observations, several bone marrow transplant teams initiated programs in which bone marrow transplantation was used to treat high-risk, poor-prognosis forms of leukemia when the disease was in complete remission.

Reported herein are the results of analyses of pooled data provided to the International Bone Marrow Transplant Registry (IBMTR), Milwaukee, by 28 transplant teams throughout the world, regarding 172 patients with acute myelogenous leukemia (AML) who were given treatment with high-dose chemoradiotherapy and bone marrow transplantation between Jan 1, 1978, and Dec 31, 1980. One hundred twenty-three patients were given grafts when their disease was in complete remission. Among the 156 patients who were given marrow from allogeic donors, 61 currently are alive one to four years after transplant; 98 have died. Seventy-two percent of these deaths occurred during the first six months after transplant. The major causes of death during this period were infection, interstitial pneumonitis, and graft-versus-host disease (GVHD). Analyses were performed to determine which of more than 20 factors occurring before and after transplant were associated with six-month mortality and with each of the three principal causes of death. The aim of this study was to detect prognostic factors that can be manipulated by referring physicians, bone marrow transplant teams, or both, so as to decrease the risk of early mortality.

CRITERIA USED TO CATEGORIZE PATIENTS

Patient Population

All cases reported to the IBMTR of patients who underwent bone marrow transplantation for AML between Jan 1,
### Table 1.—Distribution by Disease and Status of Disease for 172 Patients With AML Treated With Chemoradiotherapy and Bone Marrow Transplantation Between Jan 1, 1978 and Dec 31, 1980

<table>
<thead>
<tr>
<th>Type of Leukemia</th>
<th>Complete Remission</th>
<th>Partial Remission</th>
<th>Relapse</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First 1st to 4th</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>8 (1)</td>
<td>3</td>
<td>3</td>
<td>12 (1)</td>
</tr>
<tr>
<td>Myelogenous</td>
<td>58 (7)</td>
<td>19</td>
<td>2 (6)</td>
<td>84 (9)</td>
</tr>
<tr>
<td>Promyelocytic</td>
<td>5 (1)</td>
<td>2</td>
<td>1 (5)</td>
<td>10 (2)</td>
</tr>
<tr>
<td>Myelomonocytic</td>
<td>20 (2)</td>
<td>3</td>
<td>8</td>
<td>27 (9)</td>
</tr>
<tr>
<td>Monocytic</td>
<td>5 (1)</td>
<td>1</td>
<td>2</td>
<td>9 (1)</td>
</tr>
<tr>
<td>Erythroleukemia</td>
<td>1 (1)</td>
<td>0</td>
<td>3 (3)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>97 (12)</td>
<td>26 (0)</td>
<td>25 (2)</td>
<td>172 (16)</td>
</tr>
</tbody>
</table>

*AML indicates acute myelogenous leukemia. Numbers in parentheses indicate number of patients in that group given bone marrow from genetically identical twin donors.

### Table 2.—Interval From Date of Diagnosis Until Allogeneic Bone Marrow Transplantation

<table>
<thead>
<tr>
<th>Disease Status at Transplant</th>
<th>No. of Patients</th>
<th>Interval From Diagnosis to Transplant, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>1st complete remission</td>
<td>85</td>
<td>2.2-31.8</td>
</tr>
<tr>
<td>2nd-4th complete remission</td>
<td>25</td>
<td>6.9-58.9</td>
</tr>
<tr>
<td>Partial remission</td>
<td>22</td>
<td>1.5-52.0</td>
</tr>
<tr>
<td>Relapse</td>
<td>23</td>
<td>2.2-44.0</td>
</tr>
</tbody>
</table>

1978, and Dec 31, 1980, were included in this study; there were no exclusions. Shown in Table 1 is the distribution of patients on which this report is based. To the best of our knowledge, each team reported its consecutive bone marrow transplant experience; however, one team reported only those patients who were treated when the disease was in complete remission, and another team reported only patients given transplants between February 1978 and August 1978.

Eighty-four of the patients were male and 88 were female. The patients ranged in age from 1 to 62 years, with a median of 32.7 years; 91% were younger than 40 years at the time of transplantation. The majority of patients given transplants while in complete remission were in good clinical condition. For example, only 4% of the patients who underwent allogeneic bone marrow transplantation while in complete remission had infections at the time of transplantation. Among the patients who underwent allogeneic bone marrow transplantation while in partial remission or relapse, infections were present in 15%. In comparison, in an earlier report from the IBMTR,1 infections were present in 41% of patients with end-stage AML at the time of transplantation.

### Classification and Status of Leukemia

Usual clinical, morphological, and laboratory criteria were applied by the reporting transplant teams to establish the diagnosis of leukemia and its type.

Patients with acute undifferentiated, myelogenous, promyelocytic, myelomonocytic, monocytic, and erythroleukemia have been included in the general designation of AML. Although some groups sizes were small, there were no significant differences between the various subtypes of leukemia with respect to the survival data or the other outcome variables; therefore, for analysis the data were pooled. All patients with AML were considered to have a poor prognosis, irrespective of the status of the disease at the time of bone marrow transplantation.

The criteria summarized below were used to describe the status of the disease at the time just before initiation of pretransplant chemoradiotherapy and were based on criteria described by Cancer and Leukemia Group B.

**Complete Remission (CR).**—Five percent or fewer blasts in the bone marrow, no blasts in the peripheral blood, no hepatosplenomegaly, no lymphadenopathy, no mediastinal mass, and no gonadal or CNS involvement.

**Partial Remission (PR).**—A concentration of 5.1% to 25% blasts in the bone marrow, 1% to 5% blasts in the peripheral blood, or both, and modest hepatomegaly, splenomegaly, gonadal, or CNS involvement, with or without bone marrow and blood findings.

**Relapse.**—A concentration of 25.1% blasts or greater in the bone marrow, 5.1% blasts or greater in the peripheral blood, or both; and moderate to marked hepatomegaly or splenomegaly, with or without bone marrow and blood findings.

### Interval From Diagnosis to Bone Marrow Transplantation

The interval from diagnosis to transplant is shown in Table 2 for the 156 patients who received marrow from allogeneic donors. Among the 85 patients who received transplants when their disease was in first complete remission, 25% were given transplants within 3.8 months, 50% within 5.5 months, and 75% within 7.4 months of diagnosis.

### Chemoradiotherapy Before Transplantation

All patients received high-dose chemoradiotherapy in the ten days preceding bone marrow transplantation for leukemia cytoreduction and immunosuppression. Cyclophosphamide was given on two consecutive days to 162 (94.9%) patients at a dose of 2 mg/kg of body weight (mean, 129.0 ± 105.5 SD; range, 33 to 200 mg/kg). Additional chemotherapy including 1,3-bis-(2-chloroethyl)-1-nitrosourea, cytosine arabinoside triacetate, daunorubicin hydrochloride, doxorubicin hydrochloride, methotrexate hydrochloride, piperazinedione, prednisolone, or procarbazine hydrochloride was administered to 20 patients less than ten days before transplant. Total body irradiation (TBI) was given to 169 patients (median, 10.0 Gy; range, 9.8-12.9 Gy). A single dose of TBI was given to 152 patients and 17 patients received two to seven fractionated doses. A wide range of dose-rates of TBI was used (median, 6.6 cGy/min; range, 2.0 to 10.0 cGy/min).

### Donor-Recipient Relationships and Histocompatibility

Among the 172 patients, 156 received marrow from allogeneic donors and 16 from genetically identical twins. Of the allogeneic transplants, 156 donor-recipient sibling pairs were compatible at HL-A, HL-A, and in mixed leucocyte culture (MLC) tests; additional histocompatibility tests were performed in many instances (eg, HL-A-C, HL-A-D, HL-A-DR), and in no case did the results alter the histocompatibility relationship. Seven sibling pairs were HL-A-A and HL-A-A identical, but MLC tests were technical failures or were not performed. Thirty donor-recipient pairs (nine siblings and all four parental donors) were incompatible as determined by MLC tests; additional incompatibilities were present at HL-A-A or HL-A-B, or both, in four of these cases. Seven of the histoincompatible transplants were performed in first complete remission, three in second to fourth complete remission, and three in partial remission. There were no significant differences between the MLC-compatible, MLC-unknown, and MLC-incom-
Engraftment

Engraftment of transplanted bone marrow was classified according to criteria adopted by the Advisory Committee of the IBMTR. Sustained engraftment occurred in 116 (59%) of the 198 patients with leukemia who survived long enough for their conditions to be evaluated, ie, at least 21 days. In comparison, sustained engraftment occurred in only 76% of patients with severe aplastic anemia in a previous IBMTR report.

GVHD

Graft-versus-host disease was classified as none, mild, moderate, or severe according to the degree of cutaneous, gastrointestinal, and hepatic dysfunction. Inasmuch as there are no signs, symptoms, laboratory test results, or histological findings generally accepted as pathognomonic of GVHD, this scoring system is based, in large measure, on the judgment of the transplant team. For analysis, patients were divided into two groups: those scored as having no or mild GVHD, and those scored as having moderate or severe GVHD.

Prophylaxis of GVHD

For the purpose of evaluating the effectiveness of various regimens administered after transplant to prevent or minimize GVHD, the patients with allografts were divided into two groups according to treatment: methotrexate, 100 patients; and cyclosporin A, 51 patients. Five patients, three who received both methotrexate and cyclosporin A and two who received neither, were excluded from multivariate analyses.

STRATEGY FOR DATA ANALYSIS

The main focus of this article is on prognostic factors associated with early mortality among patients with AML who were treated with allogeneic bone marrow transplantation. The closing date for the analysis was March 31, 1982, and the minimum follow-up period was 15 months after transplant. Survival times were calculated from the date of transplant. As of March 31, 1982, ninety-five of the 156 patients with allografts had died and 63 (72%) of the 95 deaths occurred within six months following transplantation. Twenty-seven deaths occurred after six months: recurrent leukemia was responsible for 17 of these deaths. A study of factors associated with late deaths will be addressed in a separate communication from the IBMTR.

More than 20 prognostic variables occurring before and after transplant were evaluated to determine if they were associated with six-month mortality or with the principal causes of early death. Among the prognostic factors studied were age, sex of patient, sex of donor, donor-recipient sex match, disease, disease status, number of remissions, number of transfusions before or after transplant, ABO match, HLA match, MLC match, interval from diagnosis to transplant, year of transplant, presence or absence of infection at the time of transplant, pretransplant chemoradiotherapy regimen, total dose of TBI, dose rate of TBI, dose of marrow cells per kilogram of body weight, severity of GVHD, and drug used to prevent or modify GVHD.

For this study, the 156 patients with allografts were grouped according to status of disease at the time of transplantation: first complete remission, 86 patients; second to fourth complete remission, 25 patients; and partial remission or relapse, 44 patients. Survival data also are presented for the 15 patients who received marrow from genetically identical twin donors.

Statistical Methods

Initially, univariate analyses were used to screen the prognostic variables to determine whether an association existed between each of them and each of the following four outcome variables: development of infection, interstitial pneumonitis, GVHD, or death within six months. Chi square analysis (without the Yates and Bonferroni corrections) was used to test for differences between percentages. Student's t test (without correction for multiple contrasts) was used to test for differences between groups when a continuous variable (such as age or cell dose) was studied. The life-table method was used to prepare actuarial survival curves and the Lee-Deen statistic was used to test for differences between the curves. Graphical representation of the survival curves was terminated when fewer than three patients were at risk. Results from the univariate analyses were used to select factors for inclusion in multivariate analyses.

In multivariate tests, the multiple logistic function analysis was used when the outcome variable could be measured on a binary scale (eg, alive or dead, yes or no), and the Cox regression method was used when the outcome variable had a time dimension. The multivariate approach was used because it provides an estimate of the effect of each prognostic variable on each outcome variable after adjustments are made for all other variables, and because the importance of an association found in a univariate analysis may be altered when the interrelationship of other prognostic variables is taken into account.

The prognostic variables of dose rate of TBI administered before transplant and
drug administered after transplant as prophylaxis against GVHD were confounded ($r=.745, P<.0001$). Patients given a low dose rate of TBI frequently also were treated with cyclosporin A; patients given high dose rate of TBI generally also received methotrexate. In view of the fact that one or both of these prognostic factors appeared to have important associations with several outcome variables, analyses were performed twice: once with dose rate of TBI included and drug excluded, and once with drug included and dose rate of TBI excluded. The results of both analyses are presented.

In this type of report, in which many statistical tests were performed, it is possible that some results will indicate statistical significance when, in fact, the difference between groups occurred by chance. To minimize false-positive associations, we recommend that $P$ values of .01 or less be considered to be statistically significant; $P$ values between .01 and .10 are shown to indicate possible trends and to present a more complete display of the probability levels. All $P$ values are based on the results of multivariate analyses.

**RESULTS**

Presented in Fig 1 are life-table curves showing the probability of survival for 156 AML patients with allografts, grouped according to disease status at the time of transplan-
tation. The steep slope of all curves within the first six months is evident. The overall difference between the curves was significant ($P<.001$). The two-year unadjusted actuarial survival rates (and 95% confidence intervals) according to disease status were: first remission, 48% (36% to 60%); second to fourth remission, 34% (14% to 54%); partial remission, 23% (5% to 41%); and relapse, 22% (5% to 39%).

The proportion of patients who survived at least six months after transplant and the primary causes of death within six months are displayed in Fig 2. Both primary and contributory causes of death for the 58 patients with allografts who died within the first six months are shown in Table 3. Bacterial, fungal, and viral sepsis and bacterial pneumonia were the most common types of infection leading to death. Interstitial pneumonitis and GVHD were the other major causes of death within six months after transplant. In view of these findings, multivariate studies were undertaken to determine whether leads could be found that might help disclose reasons for the early deaths.

**Six-Month Survival**

The results of multiple logistic analyses testing for associations between prognostic factors and six-month survival are shown in Table 4. Patients given transplants in first complete remission who had no or mild GVHD had significantly higher six-month survival rates than those in whom moderate or severe GVHD developed. In general, patients who were treated with low rather than high dose rates of TBI and/or cyclosporin A, rather than methotrexate, also had higher six-month survival rates. It should be noted, because of confounding of dose rate of TBI with drug used to prevent GVHD, that only one of these two prognostic factors may be important.) Six-month survival rates tended to be higher among patients given few, rather than many, transfusions before transplant and among the younger patients.

**Interstitial Pneumonitis**

Interstitial pneumonitis occurred in 24% (37/156) of the patients with allografts and was lethal in 73%
Table 4.—Prognostic Factors Associated With Six-Month Survival After Allogeneic Bone Marrow Transplantation for 156 Patients With Acute Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Variables*</th>
<th>First Complete Remission</th>
<th>Second to Fourth Complete Remission</th>
<th>Partial Remission or Relapse</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>No. Alive at 6 mo./No. (%) Given Transplants</td>
<td>PT</td>
<td>No. Alive at 6 mo./No. (%) Given Transplants</td>
</tr>
<tr>
<td>GVHD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None or mild</td>
<td>42/52 (81)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Moderate or severe</td>
<td>15/32 (47)</td>
<td>&lt;.0005</td>
<td>NA</td>
</tr>
<tr>
<td>Dose-rate of TBI, cGy/min (5.5)²</td>
<td>≥2.5</td>
<td>90.4/34 (83)</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>Drug for GVHD</td>
<td>27/51 (53)</td>
<td>11/18 (51)</td>
<td>12/27 (50)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>31/36 (86)</td>
<td>7/9 (79)</td>
<td>4/6 (67)</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>24/47 (51)</td>
<td>8/17 (47)</td>
<td>12/36 (33)</td>
</tr>
<tr>
<td>No. of pretransplant transfusions (24.9)¹</td>
<td>≤24</td>
<td>33/41 (80)</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>≥25</td>
<td>21/41 (51)</td>
<td>9/10 (60)</td>
<td>11/31 (34)</td>
</tr>
<tr>
<td>Age, yr (24.2)¹</td>
<td>≤20</td>
<td>31/41 (76)</td>
<td>NS</td>
</tr>
<tr>
<td>≥24</td>
<td>26/44 (59)</td>
<td>6/15 (40)</td>
<td>7/22 (35)</td>
</tr>
</tbody>
</table>

*Prognostic variables were dichotomized to show maximum differences in outcome. Significance tests were not performed on these groupings.

†p-values are from multiple logistic function analyses and are for patients given transplants while in first complete remission. All continuous variables were analyzed as such, rather than as "bad splits."

GVHD indicates graft-versus-host disease; TBI, total body irradiation.

²Prognostic variables of dose-rate of TBI pretransplant and drug used to prevent GVHD were confounded. Therefore, multiple logistic function analyses were run twice so that both of these variables would be included in a single equation.

Numbers in parentheses are medians for continuous variables.

Table 5.—Prognostic Factors Associated With Development of Interstitial Pneumonitis (IP) Within Six Months After Allogeneic Bone Marrow Transplantation for 156 Patients With Acute Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Variables*</th>
<th>First Complete Remission</th>
<th>Second to Fourth Complete Remission</th>
<th>Partial Remission or Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Developing IP/No. (%) Given Transplants</td>
<td>PT</td>
<td>No. Developing IP/No. (%) Given Transplants</td>
</tr>
<tr>
<td></td>
<td>Dose Rate of TBI Included</td>
<td>Drug for GVHD Included</td>
<td>Dose Rate of TBI Included</td>
</tr>
<tr>
<td>Dose of bone marrow cells, X10⁷/kg (3.0)⁵</td>
<td>≤2.2</td>
<td>10/20 (50)</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>≥2.3</td>
<td>10/65 (15)</td>
<td>&lt;.0001</td>
<td>4/19 (21)</td>
</tr>
<tr>
<td>Dose-rate of TBI, cGy/min (5.5)²</td>
<td>≤5.0</td>
<td>1/3 (3)</td>
<td>&lt;.0006</td>
</tr>
<tr>
<td>≥5.1</td>
<td>10/51 (27)</td>
<td>4/13 (31)</td>
<td>12/30 (40)</td>
</tr>
<tr>
<td>Drug for GVHD</td>
<td>Cyclosporin A</td>
<td>1/3 (3)</td>
<td>&lt;.0003</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>19/47 (40)</td>
<td>5/17 (29)</td>
<td>12/36 (33)</td>
</tr>
<tr>
<td>No. of pretransplant transfusions (24.9)¹</td>
<td>≤10</td>
<td>2/16 (13)</td>
<td>&lt;.04</td>
</tr>
<tr>
<td>≥11</td>
<td>16/49 (37)</td>
<td>5/22 (23)</td>
<td>11/37 (31)</td>
</tr>
</tbody>
</table>

*Prognostic variables were dichotomized to show maximum differences in outcome. Significance tests were not performed on these groupings.

†p-values are from multiple logistic function analyses and are for patients given transplants while in first complete remission. All continuous variables were analyzed as such, rather than as "bad splits."

GVHD indicates graft-versus-host disease; TBI, total body irradiation.

²Prognostic variables of dose-rate of TBI pretransplant and drug used to prevent GVHD were confounded. Therefore, multiple logistic function analyses were run twice so that both of these variables would be included in a single equation.

Numbers in parentheses are medians for continuous variables.

(27/37) of these. No cases of interstitial pneumonitis developed more than six months posttransplant. Multivariate analysis disclosed four factors that were associated with development of interstitial pneumonitis (Table 5). Among patients given transplants in first complete remission, low doses of bone marrow cells and high dose rate of TBI or methotrexate were associated with a high incidence of interstitial pneumonitis. As reported previously by the IBMTR,⁶ because TBI dose rate and drug used to prevent GVHD were confounded, it is not clear whether one or both factors were associated with development of this complication. Also, patients given many, rather than few, pretransplant transfusions tended to have a higher incidence of interstitial pneumonitis.
### Table 6—Prognostic Factors Associated With Development of Infection Within Six Months After Allogeneic Bone Marrow Transplantation for 156 Patients With Acute Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Variables</th>
<th>First Complete Remission</th>
<th>Second to Fourth Complete Remission</th>
<th>Partial Remission or Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. With Infection/No. (%) Given Transplants</td>
<td>Dose Rate of TBI Included</td>
<td>Drug for GVHD Included</td>
</tr>
<tr>
<td>Dose rate of TBI, cGy/min (6.5)(\text{I}^{\text{I}})</td>
<td>≤(\leq)2.5</td>
<td>27/49 (55)</td>
<td>&lt;.07</td>
</tr>
<tr>
<td></td>
<td>≥(\geq)5.6</td>
<td>31/38 (86)</td>
<td>NA</td>
</tr>
<tr>
<td>Drug for GVHD</td>
<td>Cyclosporin A</td>
<td>21/36 (58)</td>
<td>NA</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>37/47 (79)</td>
<td>14/17 (82)</td>
<td>29/36 (81)</td>
</tr>
<tr>
<td>Mixed leukocyte culture match</td>
<td>Yes</td>
<td>51/77 (66)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>0/7 (00)</td>
<td>3/3 (100)</td>
</tr>
</tbody>
</table>

*Prognostic variables were dichotomized to show maximum differences in outcome. Significance tests were not performed on these groupings.
\(P\) Values are from multiple logistic function analyses and are for patients given transplants while in first complete remission. All continuous variables were analyzed as such, rather than as "best splits."
Prognostic variables of dose rate of TBI pretransplant and drug used to prevent GVHD were confounded. Therefore, multiple logistic function analyses were run twice so that both of these variables would not be included in a single equation.

### Table 7—Prognostic Factors Associated With Development of Moderate or Severe Graft–Host Disease (GVHD) Within Six Months After Allogeneic Bone Marrow Transplantation for 156 Patients With Acute Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Variables</th>
<th>First Complete Remission</th>
<th>Second to Fourth Complete Remission</th>
<th>Partial Remission or Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. With GVHD/No. (%) Given Transplants</td>
<td>Dose Rate of TBI Included</td>
<td>Drug for GVHD Included</td>
</tr>
<tr>
<td>Age, yr (24.2)(\text{I}^{\text{I}})</td>
<td>≤(\leq)20</td>
<td>11/35 (31)</td>
<td>&lt;.07</td>
</tr>
<tr>
<td></td>
<td>≥(\geq)21</td>
<td>21/49 (43)</td>
<td>NA</td>
</tr>
<tr>
<td>Dose rate of TBI, cGy/min (6.5)(\text{I}^{\text{I}})</td>
<td>≤(\leq)5.0</td>
<td>10/34 (29)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>≥(\geq)5.6</td>
<td>22/50 (44)</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td>Dose of marrow cells, (\times10^7/\text{kg}) (3.0)(\text{I}^{\text{I}})</td>
<td>≤(\leq)2.2</td>
<td>12/19 (63)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td></td>
<td>≥(\geq)2.3</td>
<td>20/65 (31)</td>
<td>8/23 (35)</td>
</tr>
<tr>
<td>Infected before transplant</td>
<td>Yes</td>
<td>2/3 (67)</td>
<td>&lt;.08</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>20/61 (33)</td>
<td>10/23 (44)</td>
</tr>
</tbody>
</table>

*Prognostic variables were dichotomized to show maximum differences in outcome. Significance tests were not performed on these groupings.
\(P\) Values are from multiple logistic function analyses and are for patients given transplants while in first complete remission. All continuous variables were analyzed as such, rather than as "best splits."

GVHD was significantly associated with an increased incidence of interstitial pneumonitis in the univariate analysis, the association was not statistically significant when other factors were taken into consideration in the multivariate analysis.

### Infection

Overall 114 (72%) of the 156 patients given allografts had infections of varying degrees of seriousness develop during the first six months after transplant; the infection led to death in 23% (26/114) of the infected patients. Multivariate analysis disclosed that the incidence of infection tended to be higher among patients transplanted in first complete remission when given high dose-rate TBI, methotrexate, or both, and in patients given marrow from MLC-incompatible rather than MLC-compatible donors (Table 6).

GVHD

Of 151 patients given allografts who survived long enough to be at risk, 62 (41%) had moderate or severe GVHD develop. This complication caused or contributed to death within six months in 35% of the 62 patients. Graft–host disease of moderate to severe intensity occurred with somewhat higher frequency among older patients, patients given high dose-rate TBI, patients given lower doses of bone marrow cells, and in patients who had infections at the time of transplantation (Table 7). It should be noted that no significant differ-
ence was found in the severity of GVHD among patients given methotrexate in comparison with those given cyclosporin A as prophylaxis against GVHD (number in whom moderate to severe GVHD developed/number of engrafted patients who survived at least 21 days: methotrexate, 42/94 [45%]; and cyclosporin A, 16/51 [31%]).

Recurrent and Persistent Leukemia

Twelve patients had recurrence of leukemia within six months after transplant. Six of the recurrences (5%) were among the 111 patients given transplants while in complete remission and six (16%) among the 45 patients given transplants while in partial remission or relapse. Overt leukemia persisted despite pretransplant chemoradiotherapy in an additional three patients and they died of leukemia within 36 days. As shown in Table 3, recurrent or persistent leukemia was responsible for only 18% (9/68) of the early deaths in the patients given allografts. In contrast, recurrent leukemia was the most frequent cause of death among patients who survived more than six months. The problem of recurrent leukemia will be the major focus of another report from the IBMTR regarding factors associated with late mortality following marrow transplantation for acute leukemia.

Histiocompatible Transplants

Among the ten patients who received histiocompatible marrow during complete remission, four are alive in complete remission, without maintenance therapy, 18 to 23 months after transplant. None of these patients given transplants while in partial remission are alive following transplantation of histiocompatible marrow (mean survival time, 40 months).

Second Transplants

Nine patients who were given transplants while in complete remission received a second transplant: five for recurrent leukemia, two for partial engraftment or no engraftment, and two for chronic GVHD. Of these nine, three are currently alive and free of leukemia, 16, 19, and 25 months after the second transplant. Two patients who were given transplants initially while in partial remission or relapse subsequently had recurrence of leukemia and received second transplants. One had transient engraftment and died of infection shortly after the second transplant. The second patient suffered a relapse after the second transplant and is receiving chemotherapy more than three years after the first transplant.

Identical Twin Transplants

Of 12 patients with AML who were given transplants while in first complete remission with marrow from syngeneic donors, nine currently are alive and free of leukemia from 15 to 33 months after transplant. Their median survival time exceeds 24 months. Three died of recurrent leukemia at ten to 14 months after transplant. An additional four patients received syngeneic marrow when their disease was in partial remission or relapse; two are alive 29 and 47 months after transplant.

COMMENT

The most important result of this study was that five factors were identified that had a strong relationship with six-month mortality or with one or more of the major causes of early death (Table 3). Use of higher dose rates of TBI was associated with a

Table 8.—Summary of Prognostic Factors Associated With Six-Month Outcome

<table>
<thead>
<tr>
<th>Prognostic Factors</th>
<th>Favorable</th>
<th>Unfavorable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strong association*</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Drug used to prevent GVHD</td>
<td>Cyclosporin A</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Dose of marrow cells</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Severity of GVHD</td>
<td>None or mild</td>
<td>Moderate or severe</td>
</tr>
<tr>
<td>Suggestive associations</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of patient</td>
<td>Younger</td>
<td>Older</td>
</tr>
<tr>
<td>No. of pretransplant transfusions</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Infected at transplant</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Donor-recipient MLC match</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

*Factors associated with end points of six-month survival, interstitial pneumonia, infection, and/or GVHD having P values of <.01 or less. Dose rate of total irradiation and drug used to prevent GVHD were confounded; only one may prove to be important.
†GVHD indicates graft-vs-host disease; MLC, mixed leukocyte culture.
‡Factors associated with end-points of six-month survival, interstitial pneumonia, infection, and/or GVHD having P values in the range between .01 and .10.
significantly higher probability of death within six months and with an increased risk of interstitial pneumonitis, infection, and life-threatening levels of GVHD. Use of methotrexate rather than cyclosporin A similarly was associated with an unfavorable six-month outcome. Because dose rate of TBI was confounded with drug used to prevent GVHD, it is not possible to state with certainty whether the rate of irradiation, the drug, or both were critical prognostic factors.

As reported previously by the IBMTR, we found that dose rate of TBI, drug used to prevent GVHD, or both, were important prognostic indicators for interstitial pneumonitis. It should be noted, however, that 117 of the 156 patients described here were included in the previous analysis. In contrast to an article by Keane et al, who reported that total absorbed lung dose of irradiation was related to death from idiopathic interstitial pneumonitis, we found no evidence to indicate that total dose of TBI within the range of 8 to 13 Gy was associated with either the incidence of or death from idiopathic or virus-associated interstitial pneumonitis. A direct comparison with the finding of Keane and colleagues could not be made, however, because the IBMTR did not have absorbed lung dose data. Larger group sizes need to be studied to evaluate fully the role of total dose, dose rate, absorbed lung dose, kinetics of repair, and fractionation of irradiation on the pathogenesis of interstitial pneumonitis.

A new finding in the present report was that the dose of bone marrow cells also had a significant association with interstitial pneumonitis. It is possible to speculate that the lower doses of bone marrow cells resulted in delayed restoration of immune competence with increased susceptibility to the disease. Although age was not significantly associated with interstitial pneumonitis in the multivariate analysis, it may have played a role, because younger patients tended to receive higher doses of bone marrow cells per kilogram of body weight, while cell doses were lower in older patients. The mean cell doses per kilogram of body weight by age quartiles were: 1 to 15 years, 3.8; 16 to 24 years, 2.9; 25 to 32 years, 2.7; 33 to 62 years, 2.6, and the two factors were significantly correlated.

In a study of 67 patients with aplastic anemia, leukemia, and lymphoma who received allografts, Ramsey et al found a highly significant association between the recipient's age and occurrence of acute GVHD. Furthermore, they observed no cases of GVHD in approximately 17 patients younger than 12 years. Although the incidence of life-threatening GVHD in the present report tended to be lower in younger patients (Table 7), it still represented a formidable problem. Thus, among those at risk, moderate or severe acute GVHD occurred in 41% of 39 patients aged 16 years or older and in 24% of 17 patients younger than 12 years.

Fewer than 50% of the patients in whom moderate or severe GVHD developed following bone marrow transplantation during the first complete remission survived for six months (Table 4). Shown in Fig 3 is a life-table analysis comparing overall survival of patients in whom moderate or severe GVHD developed with those in whom no or mild GVHD developed. The difference in the survival curves was significant (P<.005) and emphasizes the importance of developing improved methods to prevent, circumvent, or treat GVHD.

The observation that the dose of bone marrow cells per kilogram of body weight tended to be inversely proportional to the severity of GVHD (Table 7) was contrary to observations in other mammalian systems. One possible explanation to account for this difference may have been that recipients who were given lower cell doses had donors from whom marrow was difficult to aspirate, and, as a consequence, more mature T cells were present in the inoculum due to admixed peripheral blood.

Unfavorable outcomes tended to occur in older patients, patients given many pretransplant transfusions, patients who acquired infections at the time of transplantation, and the presence of MLC incompatibility between donor and recipient (Table 8).

As shown in Figs 1 and 2, an additional prognostic factor that showed a significant association with six-month outcome was status of the disease at the time of transplantation: the more advanced the disease, the higher the mortality rate (P<.001).

The strong and suggestive prognostic factors found in this study can be divided into two groups—those that often can be controlled by the referring physician or by the transplant team and those that are difficult or impossible to control. For example, the severity of GVHD and the age of a patient requiring bone marrow
transplantation are difficult factors to control. On the other hand, the status of the disease at transplant, the dose rate of TBI, the drug selected to prevent GVHD, the dose of bone marrow cells administered, and the number of pretransplant transfusions often can be manipulated.

A controlled clinical trial would be the best mechanism to evaluate the importance of the favorable and unfavorable prognostic factors found in the present study (Table 8). Knowledge that associations were found between these factors and six-month outcome should be helpful in planning a prospective study. In those situations when it is difficult to conduct a controlled clinical trial within a reasonable period of time, pooled data from numerous transplant centers (as reported to the IBMTR), can be used to simulate such a trial. For example, presented in Fig 4 is a life-table analysis showing survival among the patients with allografts reported here who were given transplants while in first complete remission. The patients were selected for inclusion on the basis of two prognostic factors from Table 8 that often can be manipulated: number of pretransplant transfusions and dose of bone marrow cells administered. The upper curve represents patients given fewer than 25 transfusions and at least 2.3x10^10 bone marrow cells per kilogram of body weight. The lower curve represents patients given more blood transfusions and fewer bone marrow cells. The magnitude of the difference between these curves (or between curves in life-table analyses evaluating other prognostic factors) should prove helpful in estimating the number of patients needed to be entered into a controlled clinical trial to detect a significant effect of the variables to be studied.

Finally, it should be noted that two-year disease-free survival was achieved in an impressive proportion of patients who had suffered relapses one or more times while receiving chemotherapy, or whose disease could not be brought into complete remission with chemotherapy (Fig 1). This suggests that bone marrow transplantation should be considered for all patients with advanced AML if they are of appropriate age and have an HLA-identical donor available.

LIMITATIONS OF THIS REPORT

We wish to emphasize that the data reported herein were not derived from a controlled clinical trial. Inherent in this type of study, based as it is on data from 28 different centers, is the fact that there were many unrecorded factors and differences in medical practice that may have influenced outcome. To a large extent, differences between centers were due to differences in experience, available support services, and sophistication of the teams. Inasmuch as bone marrow transplantation is still an evolving therapeutic procedure, most teams follow their own unique protocols. Although this contributes to the variability between centers, the differences represent an important strength of the IBMTR. When significant prognostic factors are uncovered, we believe that they are usually conservative estimates, because the random variation between centers tends to obscure significant differences. Thus, factors reported here that showed significant associations often confirmed reports of others or, when previously undescribed associations were found, they likely will be confirmed when more data became available.

Another consideration was that there were distinct differences in the number of cases submitted by the various transplant centers. For example, 11 of the 28 teams submitted only one case each, whereas a single transplant center contributed data for 50 cases, or 29% of the total patients in this study. The next highest contributing center submitted data for 20 patients (12%). The largest center contributed data for most of the patients who were treated with TBI at dose-rates of 5.5 Gy/min or less. With this exception, there were no differences in the results reported here when analyses were performed with or without inclusion of their data.

The Advisory Committee of the IBMTR is concerned that data provided to the IBMTR are reported in a uniform manner. To address this problem, guidelines for uniform reporting of data are distributed periodically to all transplant teams. Nonetheless, problems do exist. For example, what is reported by pathologists at one center as 4% blasts in the marrow (thus fulfilling a criterion for complete remission) could be reported as 6% blasts (and classified as partial remission) at another center. This type of "borderline" classification does not constitute a serious difficulty because multiple criteria are used to reduce the possibility of bias in scoring critical end points, such as status of disease at the time of transplantation or severity of GVHD.

RECOMMENDATIONS OF THE ADVISORY COMMITTEE

In view of the present controversy regarding the relative merits of chemotherapy v bone marrow transplantation for patients with AML in first complete remission, the Advisory Committee of the IBMTR recommends that a prospective and randomized clinical trial be planned to compare these two therapeutic modalities under carefully controlled conditions. Furthermore, it is recommended that the findings reported here be taken into consideration in planning such a study.

The research on which this publication is based was supported by grants from the Jacob and Hilda Blaustein Foundation; Burroughs-Wellcome Fund; Commission of the European Communities; Charles E. Colpeter Foundation; Elizabeth Esher Dochtart Charitable Trusts; Carl and Elizabeth Eberbach Foundation; March of Dimes-Birth Defects Foundation; Ambrose Monell Foundation; Mount Sinai Medical Center (Miami Beach); Samuel Roberts Noble Foundation; Queen Wilhelmina Fund; Sandos Limited; Swim Cancer League; and the Upjohn Company, and was conducted under contracts NOI-AI-00564 and NOI-AI-26903 from the National Institute of Allergy and Infectious Diseases and the National Cancer Institute, US Department of Health and Human Services.

Jill R. Parmer provided help with data analysis and Elaine L. Koba and D'Etta Waldock provided help with preparation of the manuscript.

The bone marrow transplant teams at the following institutions contributed the patient data that was the basis for this report: It is hoped that data provided to the IBMTR by bone marrow transplant teams throughout the world in a spirit of international cooperation will accelerate understanding of the critical factors necessary for successful treatment of patients who might be benefited by bone marrow transplantation.

Institutions Contributing Patient Data for This Report

Academisch Ziekenhuis Leiden, Leiden, the Netherlands; Christchurch Hospital, Christchurch, New Zealand; Ciudad Sanitas, Valencia, Spain; Cleveland Clinic, Cleveland; Comprehensive Cancer Center, Rotterdam, the Netherlands; Hôpital Saint Louis, Paris; Hospital of Fossaria, Pescara, Italy; Huddinge University Hospital, Huddinge, Sweden; Kanazawa University School of Medicine, Kanazawa-shi, Japan; Kantonsspital Basel, Basel, Switzerland; Kan-


PATHOGENESIS OF INTERSTITIAL PNEUMONITIS FOLLOWING ALLOGENEIC BONE MARROW TRANSPLANTATION FOR ACUTE LEUKEMIA

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ABSTRACT Interstitial pneumonitis (IPn) is one of the major causes of treatment failure following allogeneic bone marrow transplantation. Its cause, prevention and treatment are unknown. Data from individual transplant centers and from the International Bone Marrow Transplant Registry (IBMTR) indicate that one of every four patients with acute leukemia treated with marrow allografts died of IPn. A model was formulated to demonstrate possible pathways for the pathogenesis of the disease.

INTRODUCTION

Interstitial pneumonitis is an extremely grave complication of allogeneic bone marrow transplantation. Data from the IBMTR indicate that 29% (130/450) of patients with acute leukemia developed IPn following

1This work was supported by grants from the Jacob and Hilda Blaustein Foundation, Burroughs-Wellcome Fund, Culpeper Foundation, Lederle Laboratories, Ambrose Monell Foundation, Noble Foundation, Sanofi Ltd., Swiss Cancer League, Upjohn Company, and was performed pursuant to Contracts NO1-AI-02648 and NO1-AI-22669 from the National Institutes of Allergy and Infectious Diseases and the National Cancer Institute, USDHHS and Contract B10/C.520.Us(H) from the Commission of the European Communities.
allogeneic marrow transplantation; the case fatality rate was 69% (90/130) and the mortality rate was 20% (90/450) (manuscript in preparation). In another large series of allografted patients with acute leukemia, Meyers et al. (1) reported an incidence of 47% (153/323), a case fatality rate of 68% (104/153) and a mortality rate of 32% (104/323). The problems are: a.) The cause is not known; b.) No effective preventive measures are known; c.) No effective treatment is known; and d.) At the present time approximately 25% of allografted leukemic patients die of IPn.

In this review, a model is presented to help describe interacting factors believed to be involved in the pathogenesis of IPn. Although the emphasis is on IPn in leukemic patients, at least some of the mechanisms discussed also apply to IPn in marrow allografted patients with primary immunodeficiency disease and severe aplastic anemia, as well as to IPn in general.

PATHWAYS FOR THE PATHOGENESIS OF INTERSTITIAL PNEUMONITIS

To plan effective preventive measures, it is necessary to understand the many complex interacting factors that lead to the development of IPn. A model is presented (Fig. 1) to help sort out these factors. Basic to this model is the concept that there are three major predisposing factors for the development of IPn; a.) an immunosuppressed host; b.) lung damage; and c.) the presence of opportunistic microorganisms (1-13). Any of these three predisposing factors alone can lead to the development of IPn; the three in combination can be likened to a time bomb ready to explode.

Immunovulnerable Host

There is a sequence of pre- and posttransplant events that contribute toward establishment of an immunologically crippled host who, as a consequence, is vulnerable to infection (Fig. 1). For example, acute leukemia at the time of diagnosis often is advanced, debilitating and immunosuppressive. Remission-induction and consolidation chemotherapy given for weeks or months before transplantation has immunosuppressive activity. The pretransplant
Figure 1. Possible pathways for the pathogenesis of interstitial pneumonitis
conditioning regimen has profound immunosuppressive effects. Cyclophosphamide plus total body irradiation (TBI) is the usual basic conditioning regimen, to which other antileukemic drugs having immunosuppressive side effects often are added. Posttransplant immunosuppression with methotrexate, cyclosporin-A, antithymocyte globulin and other immunosuppressive agents, given to prevent, modify or treat graft-vs-host disease (GVHD), adds to and prolongs the immunosuppressed state of the patient. Finally, GVHD, per se, has immunosuppressive effects (14).

Shown in Table 1 is a list of agents that are commonly administered to patients with acute leukemia to induce or maintain remission and/or for immunosuppression in the peritransplant period. Most of these agents have immunosuppressive characteristics; as described below, all have been reported to cause lung damage and to be associated with IPn (15-24).

Thus, as shown in Fig. 1 and Table 1, allografted leukemic patients have multiple and cumulative interacting factors that result in virtual destruction of a major host defense against infection. The fact that opportunistic pathogens often are found in the lungs of patients with IPn suggests that the severe and prolonged injury to the immune apparatus may allow growth of microorganisms, especially in lungs that are damaged (1,11).

<table>
<thead>
<tr>
<th>Agents Associated with Interstitial Pneumonitis</th>
<th>That Are Immunosuppressive and/or Cause Lung Damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin</td>
<td>Irradiation&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BCNU</td>
<td>Methotrexate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bleomycin</td>
<td>Melphalan</td>
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<tr>
<td>Busulfan</td>
<td>Mitomycin</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>Procarbazine</td>
</tr>
<tr>
<td>Cyclophosphamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Vincristine</td>
</tr>
</tbody>
</table>

<sup>a</sup>Frequent association
Lung Damage

The same sequence of events that leads to immunosuppression also has direct and cumulative effects that injure the lungs (Fig. 1). For example, leukemic infiltrates occasionally appear in the lung and cause localized damage. All the drugs shown in Table 1 have been reported to cause lung damage and to be associated with IPn. Perhaps of greater importance is the observation that several of these drugs were reported to interact with irradiation in such a way as to increase the amount of injury to normal lung tissues (25,26). This observation is of particular relevance to the problem of IPn in allografted leukemic patients. At the present time, most leukemic patients who undergo bone marrow transplantation are treated with cyclophosphamide and/or methotrexate along with TBI; lung damage has been reported to be magnified when either of these drugs was administered in conjunction with lung irradiation (25,26).

Impairment in function and delayed recovery of alveolar macrophages was found in marrow allografted patients (27) and dogs (28) that had been treated with cyclophosphamide, TBI and methotrexate. Inasmuch as pulmonary alveolar macrophages have been shown to contain CMV virus particles during CMV infections, it was suggested that these cells may represent a host defense mechanism against CMV (29). Thus, the combination of increased lung damage and impaired alveolar macrophage function may explain the fact that among patients treated with cyclophosphamide and TBI pretransplant, the incidence of IPn was significantly higher when methotrexate rather than cyclosporin-A was administered posttransplant (4,5).

Although it is well known that irradiation injures the lungs (8,30), the precise role of TBI in the pathogenesis of IPn has not yet been identified clearly. Key questions are the extent to which a.) the dose-rate of TBI, b.) use of fractionated TBI or c.) the total dose to the lung affects the risk of IPn in allografted leukemic patients. Clinical studies at various centers are in progress to try to answer these questions.

Barrett et al. (31) first suggested that the dose-rate of TBI might be an important factor in the pathogenesis of IPn. Subsequently, multivariate analyses of data reported
to the IBMTR disclosed a strong association between dose-rate of TBI and IPn, irrespective of the total dose of irradiation to the lungs (4-6). Thus, the incidence of IPn among patients given TBI at a dose-rate < 6.0 cGy per minute was 6% (4/69), whereas the incidence was 30% (32/107) when the dose-rate was higher (5). This observation has not, as yet, been confirmed in prospective studies.

The use of fractionated rather than single dose TBI was associated with a reduction in the incidence of IPn from 70% to 33% and a reduction in the case fatality rate from 50% to 23% in one study (10). The total dose was 13.2 GY, dose to lungs 9.0 GY (lung shielding), 11 fractions administered over four days, dose per fraction 1.2 GY and dose-rate 10-19 cGy per minute. In contrast, Meyers, et al. (1) found no beneficial effect in a multivariate comparison of allografted leukemic patients treated with cyclophosphamide plus single dose TBI vs cyclophosphamide plus fractionated TBI. Their incidence of IPn with single dose was 51% (84/165) and with fractionated, 37% (30/82). There was no indication in their report, however, whether the radiobiological effect of the single dose and the total of the fractionated doses were comparable. Clift, et al. (32) reported a high incidence (44%) of IPn and/or alveolar pneumonitis when fractionated TBI was used in patients with acute lymphoblastic leukemia in relapse. Six different regimens for 41 patients were evaluated with total doses ranging from 12.0-17.5 GY, six or seven fractions of 1.0-4.0 GY per fraction administered over two to seven days at a dose-rate of 3-8 cGy per minute.

It is possible that the conflicting reports on the effect of fractionated TBI on the incidence of IPn might be due to differences in the dose per fraction in different centers (range 1.0-4.0 GY per fraction). To cause a marked reduction in the risk of IPn, it may prove necessary to reduce the dose of TBI to < 1.0 GY per fraction in order to allow repair of irradiation-induced lung damage.

Another approach to spare the lungs from damage caused by ionizing irradiation has been to restrict lung doses to < 7.5 GY by shielding the lungs with lead during TBI, or by limiting the dose of TBI to < 7.5 GY. Preliminary analysis of data reported to the IBMTR gave no clear indication that this approach was associated with a reduced risk of IPn (manuscript in preparation).
Finally, while the lung is not considered to be a primary target organ of the graft-vs-host reaction, lung damage and decreased pulmonary function have been reported to be associated with both acute and chronic GVHD (33,34).

Thus, as shown in Fig. 1 and Table 1, allografted leukemic patients have multiple and cumulative interacting factors that cause injuries to the pulmonary parenchyma and to alveolar macrophages. The fact that fungal and viral microorganisms often are found in the lungs of patients with IPn also suggests that damaged lungs provide a situs for growth of opportunistic pathogens.

Opportunistic Microorganisms

A partial list of nonbacterial microorganisms reported to be associated with IPn is presented in Table 2. Cytomegalovirus (CMV) was the pathogen found most frequently in the lungs of allografted leukemic patients with IPn (1,3,12,35, and unpublished ISMTR data). Pneumocystis carinii often was associated with IPn in the past (2,11,36), but since the introduction of trimethoprim-sulfamethoxazole prophylaxis, the problem has all but been eliminated from bone marrow transplant recipients (1,12). It should be borne in mind, however, that the finding of opportunistic microorganisms such as CMV, Pneumocystis, etc. in the lungs of patients with IPn does not prove that the organism is the causative agent. Damaged lungs in immunosuppressed patients with IPn may simply provide a suitable ecologic niche for overgrowth of opportunistic pathogens.

If one assumes that CMV is, in fact, important in the pathogenesis of IPn, then the question of its mode of entry is of considerable import and has generated numerous studies. Activation of a latent CMV infection is one possible route (Fig. 1). Neiman, et al. (35) found that 45% of 80 marrow allografted patients had significant antibody titers against CMV pretransplant and were presumed to have had previous exposure to the virus. GVHD, antileukemic and immunosuppressive agents have been shown to be capable of activating latent CMV and other herpes viral infections (37-39). Their cumulative effects (Fig. 1) could readily account for activation of endogenous microorganisms. For these patients without previous viral
or fungal infections, airborne infection and the frequent requirement for transfusion of blood products from multiple donors pre- and posttransplant are other potential sources of these microorganisms (40–42).

Thus, there is no shortage of endogenous or exogenous sources of CMV and other ubiquitous agents as opportunistic pathogens for IPn. The presence of lung damage in an immunovulnerable host strengthens the hypothesis that these microorganisms are, indeed, the etiologic agents and that infection is responsible for many cases of IPn. On the other hand, from approximately 30–50% of IPn cases in allografted leukemic patients are, of necessity, categorized as idiopathic (1,29 and unpublished IBMTR data). Despite the use of careful and thorough microbiological examinations of biopsied lung tissue and, all too often, tissue obtained postmortem, no pathogen could be identified in many cases.

Other Considerations

Mentioned below are some unexplained and intriguing observations that, when clarified, may improve our understanding of the pathogenesis of IPn.

Isografts. In a series of 100 patients that were transplanted with syngeneic marrow for aplastic anemia or hematologic malignancy, Applebaum, et al. (43) found that the incidence of IPn was 17%, but there were no cases of

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<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Etiologic Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces</td>
<td>Herpes Simplex</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Histoplasma</td>
</tr>
<tr>
<td>Candida</td>
<td>Mycoplasma</td>
</tr>
<tr>
<td>Coccidioides</td>
<td>Pneumocystis</td>
</tr>
<tr>
<td>Cryptococcus</td>
<td>Toxoplasma</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Varicella Zoster</td>
</tr>
</tbody>
</table>

*Frequent association*
CMV-associated IPn. In contrast, CMV was associated with IPn in 16% (84/520) of similar patients who were given marrow from allogeneic donors (1). The incidence of idiopathic IPn was similar in the isografted and allo-grafted patients. The three main differences were that one group received marrow from identical twins and the other from allogeneic donors; the isografted patients did not receive methotrexate posttransplant, whereas the allo-grafted patients did; and there (presumably) was no host-vs-graft or graft-vs-host reaction in the isografted group. As a consequence of these confounding variables, the authors were unable to identify the mechanism to explain the different risks of CMV-associated IPn in their isografted vs allografted patients (43).

GVHD. Although, as discussed above, GVHD was shown to contribute toward immunosuppression, lung damage and activation of endogenous CMV, multivariate analyses disclosed only borderline associations between the intensity of GVHD and the development of IPn (1,4,5). When taking the isograft findings into account (43), one interpretation of these findings suggests that GVHD plays a relatively minor role in the pathogenesis of idiopathic IPn, but that it may play an important role in activating CMV and, thus, in the pathogenesis of CMV-associated IPn.

Yearly Incidence. Chronologically there has been no significant decrease in the annual incidence of IPn (1) and, as a matter of fact, the incidence may be increasing (unpublished IBMTR data). This is true despite the fact that the risk of Pneumocystis-associated IPn has declined (1,12).

Age. Younger patients were found to have a lower incidence of IPn than older patients (1). The reason for this difference is obscure.

Sex-match. A recent report from the IBMTR regarding 176 allografted leukemic patients disclosed that the sex-match of donor and recipient was significantly associated with the development of IPn (5). In this multivariate study, female patients who received cells from female donors had a 32% incidence of IPn while male patients given female cells had a 44% incidence; the incidence was intermediate when cells from male donors were transplanted into male (17%) or female (18%) recipients. The difference in incidence between female-female donor/recipient pairs and the other sex-match combinations was not due to an increased level of GVHD in the latter groups (5). The
association between sex-match and the risk of IPn has not been confirmed by others and the reason for the significant differences between groups is not known.

Cell Dose. Administration of low doses of bone marrow cells was found to be associated with a high risk of IPn among 85 patients with acute myelogenous leukemia who were transplanted in first complete remission (4). The explanation for this finding also is unknown.

STRATEGIES TO PREVENT INTERSTITIAL PNEUMONITIS

At the present time, use of trimethoprim-sulfamethoxazole prophylaxis has reduced the incidence of Pneumocystis-associated IPn (1,12), but its use has not resulted in a reduction in the overall incidence of IPn. Thus far, there have been no clear indications in reports from individual transplant centers of other measures that are effective at preventing IPn.

Multivariate analyses of IBMTR data identified four factors that were significantly associated with the risk of IPn (4-6). It is hoped that this knowledge will point the way for effective preventive measures against the development of IPn. Identification of these factors was based on retrospective analyses rather than on prospective controlled clinical trials. As yet, none of the associations between these factors and the risk of IPn has been confirmed by individual transplant centers. On the other hand, univariate analyses of an expanded IBMTR database (450 patients with acute leukemia transplanted with marrow from HLA-A, B, and MLC identical allogeneic siblings between January, 1977 and May, 1982) appear to substantiate the earlier IBMTR reports (4-6). It is not possible on the strength of these findings to recommend that individual bone marrow transplant centers change their protocols on the basis of these statistical associations because of the many differences in practice between centers that in some instances could not be identified in the IBMTR analyses. It is recommended, however, that these variables be taken into consideration in planning controlled clinical trials.
Dose-rate of TBI

The incidence of IPn was low when the dose-rate of TBI was low (4-6). The most plausible explanation for this observation is that low dose-rate irradiation allowed repair of normal tissues from the radiation injury. Additional time for repair would occur among patients given low dose-rate TBI because of an increased number of interruptions during irradiation to provide nursing care, turn the patient, etc. than during high dose-rate TBI. In these studies, however, the variables of dose-rate of TBI and the drug used to prevent or modify GVHD were confounded (4-6). It was not possible to determine whether one or both of these prognostic factors was important.

Drug Used to Prevent GVHD

The risk of IPn was low when cyclosporin-A was used as prophylaxis against GVHD, but high when methotrexate was used (4,5). The reason for the difference was unclear. If one assumes that this variable is independent of the dose-rate of TBI and important on its own, then it is possible that the interactions of cyclophosphamide, TBI and methotrexate, in comparison with cyclophosphamide, TBI and cyclosporin-A, resulted in greater cumulative damage to the immune system and the lung and had a greater propensity for activation of endogenous microorganisms. Alternatively, cyclosporin-A may have some as yet unknown salutary effect that allows recovery of an important immune defense, promotes healing of the damaged lung or has an antimicrobial effect.

Sex-match

The IBMTR data suggested that with all other things being equal and given the opportunity, female patients should receive marrow from female rather than male donors (5). The reason for the increased incidence of IPn in male patients who received marrow from male donors and in recipients who received marrow from sex-mismatched donors is unclear.
Bone Marrow Cell-dose

In a multivariate study, patients given relatively low doses of bone marrow cells per kg body wt had an increased incidence of IPn in comparison with patients given higher doses (4). This finding would suggest that for the prevention of IPn, larger rather than smaller doses of bone marrow cells should be given. However, the group sizes were small, the effect of cell-dose on other end-points has not been studied thoroughly, and the reason for the association is not understood. Therefore, with this possible preventive measure, as well as the others mentioned, additional studies are necessary.

SUMMARY

Dividends are beginning to be realized from analyses of data from the burgeoning number of leukemic patients being treated with high-dose chemoradiotherapy and allogeneic bone marrow transplantation. Some of the mysteries regarding the interrelationships between factors present in the peritransplant period and the pathogenesis of IPn are beginning to be unraveled. A model was presented to help clarify some of these interactions (Fig. 1). Three critically important predisposing factors have been recognized: damage to the lungs, immunovulnerability of the host, and the frequent presence of opportunistic pathogens. Discussion was focused on some of the issues that need to be resolved in order to prevent this terrible complication of bone marrow transplantation.

Everyone involved with clinical bone marrow transplantation recognizes the magnitude of the IPn problem. Transplant teams are encouraged to report their data to the IBMTR for pooling and analysis in an effort to help solve the problem. Also, randomized controlled clinical trials urgently are needed to investigate methods to reduce the risk of IPn in prospective studies.

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Hilbert and Ms. Patricia J. Minks for their help in 
preparation of this manuscript.

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Hyperfractionated total body irradiation for bone


Interstitial Pneumonitis After Bone Marrow Transplantation
Assessment of Risk Factors

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Data from 932 patients with leukemia who received bone marrow transplants were analyzed to determine factors associated with an increased risk of developing interstitial pneumonitis. Interstitial pneumonitis developed in 268 patients for a 2-year actuarial incidence of 35 ± 4% (SD) and with a mortality rate of 24%. Six factors were associated with an increased risk: use of methotrexate rather than cyclosporine after transplantation (relative risk, 2.0; p < 0.0002); presence of severe graft-versus-host disease (relative risk, 1.5; p < 0.0003); long interval from diagnosis to transplantation (relative risk, 1.6; p < 0.002); performance ratings before transplantation of less than 100% (relative risk, 2.1; p < 0.0001); and high dose-rates of irradiation in patients given methotrexate after transplantation (relative risk, 3.2; p < 0.03). The risk of developing interstitial pneumonitis ranged from 8% in patients with none of these adverse risk factors to 94% in patients with all six. These findings may help to identify patients at high risk for this complication.

Bone marrow transplantation from an HLA-identical sibling is an important treatment for leukemia (1-7), aplastic anemia (8, 9), severe combined immunodeficiency (10), and other disorders (11-13). Long-term, disease-free survival is now achievable in approximately 50% of these patients. Despite these advances, several transplantation-related complications have limited the further success of this technique. Most prominent among these are graft-versus-host disease (14, 15) and interstitial pneumonitis (16-18).

Interstitial pneumonitis accounts for more than 40% of transplantation-related deaths in most large series. Patients with this complication develop dyspnea, fever, nonproductive cough, and hypoxia. Roentgenograms of the chest typically show bilateral interstitial infiltrates, and ventilatory tests show decreased diffusion capacity. Histologic examination of the lung shows edema, fibrosis, a variable cellular infiltrate, and alveolar exudates (19-21).

The onset of interstitial pneumonitis characteristically occurs within 90 days after bone marrow transplantation. Reported incidence rates are highly variable, ranging between 10% and 84% in several series (16-19, 22, 23). The reason for this variability may be related to different definitions of the disease (19-21), different diagnostic criteria, and, most importantly, the relatively small number of patients studied at most centers.

Cytomegalovirus, Pneumocystis carinii, herpes simplex virus, herpes zoster virus, Aspergillus, Candida, and other infectious agents have been isolated from the lungs of patients with interstitial pneumonitis, either by open lung biopsy or at autopsy. Reports from individual centers suggest that interstitial pneumonitis is associated with cytomegalovirus infection in 30% to 70% of the cases. Pneumocystis infections, once common, are now rare, probably because of the prophylactic use of trimethoprimsulfamethoxazole (16). Other viral, protozoal, and fungal infections occur less frequently; in some instances more than one organism has been isolated. No pathogenic organism is identified in up to 60% of the cases, which are referred to as idiopathic. It is likely that some of these cases represent undiagnosed infections, particularly with cytomegalovirus, because techniques to isolate this virus require greater sophistication than is available at some centers. Other cases may result from drug or radiation toxicity or possibly from immune reactions involving the lung.

There have been several analyses of factors associated with the development of interstitial pneumonitis (17-19, 22-25). Factors reported to be predictive include prior infection of the recipient or donor with cytomegalovirus, lung dose or dose-rate of irradiation, sex-matching of donor and recipient, exposure to chemotherapy before transplantation, dose of bone marrow cells, number of transfusions before transplantation, granulocyte transfusions after transplantation, drug used to prevent or treat graft-versus-host disease, year of transplantation, and so forth.

From the International Bone Marrow Transplant Registry and the Division of Medical Oncology and the Bone Marrow Transplantation Program, University of Florida College of Medicine, Gainesville, Florida; Department of Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin; Department of Medicine, Division of Hematology-Oncology, UCLA Center for Health Sciences, Los Angeles, California; Service d'Hematologie, Hopital St. Louis, Paris, France; Department of Clinical Pathology, Royal Marsden Hospital, London, England; Institut für Hämatologie, Musich, West Germany; Division of Hematostasis/Clinical Epidemiology, Medical College of Wisconsin, Milwaukee, Wisconsin.

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others. Most reports have been controversial, with different centers reporting disparate results.

Because of these contradictory reports and the importance of accurately predicting the occurrence of interstitial pneumonitis in patients, we analyzed data from 932 patients with leukemia who received HLA-identical bone marrow transplants between 1978 and 1983. We identified six factors that appear to predict independently the development of interstitial pneumonitis.

Patients and Methods

PATIENTS

Comprehensive data were reported to the International Bone Marrow Transplant Registry by 69 transplant centers worldwide for 1183 consecutive patients with leukemia who received bone marrow transplants between 1 January 1978 and 30 June 1983. The minimum follow-up time was 7 months. Data from the 932 patients who received transplants from HLA-A, B-, and mixed-leukocyte-culture-identical sibling donors with no evidence of histoincompatibility at other HLA loci are the basis for this study. Forty-six of these six patients had acute myelogenous leukemia, 339 had acute lymphocytic leukemia, and 187 had chronic myelogenous leukemia. Characteristics of patients before transplantation are presented in Table 1. Univariate analyses showed that neither diagnosis nor disease status was significantly associated with the occurrence of interstitial pneumonitis. As a consequence, subsequent analyses combined all 932 patients.

Most patients (920 of 932) were prepared for transplantation with both immunosuppressive-antileukemic cytotoxic chemotherapy and total body irradiation. After transplantation, patients were given methotrexate, cyclosporine, corticosteroids, antithymocyte globulin, or other agents, alone or in combination, to prevent or treat graft-versus-host disease. In this report, interstitial pneumonitis includes any nonmalignant pneumonia with radiographic or histologic features commonly associated with the disease (19-21). Biopsy or autopsy material was studied from more than 75% of patients.

Statistical Methods

Thirty-three potential prognostic variables were studied (Table 2). The association between each factor and the occurrence of interstitial pneumonitis was tested with chi-square and t tests. Factors found to be significantly associated with interstitial pneumonitis by these univariate tests were included in a multiple logistic equation. From the logistic analysis, we derived the probability of interstitial pneumonitis for a patient with a single risk factor or a specific profile of risk factors. The relative risk associated with a single risk factor or profile was computed as the probability of disease for patients with that risk factor or profile divided by the probability of disease for patients with none of the risk factors.

To determine whether risk factors differed according to the type of disease, we did separate univariate and logistic analyses for patients with interstitial pneumonitis that was idiopathic or associated with cytomegalovirus. Differences between treatment centers that could not be accounted for by recorded information were tested in two ways in logistic analysis after we adjusted for the known risk factors: first, by comparing the group of patients given transplants in smaller centers with an approximately equal-sized group given transplants in larger centers; and second, by comparing rates of interstitial pneumonitis in patients from each of the seven largest centers with rates in the remaining patients. The actuarial incidence of interstitial pneumonitis was computed by standard life table methods (26).

Results

Two hundred sixty-eight patients developed interstitial pneumonitis for an actuarial incidence at 2 years of 35 ± 4% (SD). The median time from transplantation to onset of interstitial pneumonitis was 52 days (range, 2-49); in 71% of the patients the disease occurred within 90 days, and in 91% it occurred within 180 days after transplantation. Interstitial pneumonitis was the primary cause or a contributory cause of death in 224 patients, for a case-fatality rate of 84%.

Table 2. Peritransplant Factors Tested for Possible Associations with Interstitial Pneumonitis

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Interval diagnosis to transplantation</td>
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<tr>
<td>Dose-rate of total body irradiation</td>
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<tr>
<td>Drug to prevent graft-versus-host disease</td>
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<tr>
<td>Dose of cyclophosphamide before transplantation</td>
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<tr>
<td>Dose of total body irradiation</td>
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<tr>
<td>Lung dose of irradiation</td>
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<td>Fractionated irradiation</td>
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<td>Number of fractions of irradiation</td>
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<td>Antibiotic decontamination</td>
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<td>Type of isolation</td>
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<td>Trimethoprim-sulfamethoxazole prophylaxis</td>
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<td>Bone marrow cell dose</td>
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<tr>
<td>ABO match</td>
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<tr>
<td>Previous splenectomy</td>
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<td>Infected at transplantation</td>
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<td>Irradiation source</td>
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<td>Disease</td>
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<td>Coexisting disease</td>
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<td>Performance rating before transplantation</td>
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<td>Alloimmunized donor</td>
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<td>Severity of graft-versus-host disease</td>
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* By the referring physician or the transplant team.

Table 1. Characteristics of Patients with Leukemia Before Transplantation

<table>
<thead>
<tr>
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<tr>
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<tr>
<td>Interval from diagnosis to transplantation,</td>
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<tr>
<td>mos</td>
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<tr>
<td>Performance rating, %</td>
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<tr>
<td>Disease status at transplantation, %</td>
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<tr>
<td>First complete remission or chronic phase</td>
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<td>Infection present at time of transplantation,</td>
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<td>%</td>
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<td>Organ impairment at time of transplantation,</td>
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<td>%</td>
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</tbody>
</table>

* Values given with ranges (in parentheses) are medians.
* Each unit of platelets was counted as one transfusion.
presence of severe graft-versus-host disease (Figure 1C),
performance ratings (27) before transplantation of less
than 100% (Figure 1D), and increased time from diag-
nosis to transplantation (Figure 1E). Dose-rates of irra-
diation of 6.0 cGy/min or more were associated with
an increased incidence of interstitial pneumonitis in the 672
patients who received methotrexate for prophylaxis
against graft-versus-host disease (Figure 1F). A dose-
response trend was apparent at lower dose-rates, but a pla-
teu was reached at 6.0 cGy/min with no further incre-
ment in the risk of disease with dose-rates up to 108 cGy/
min. This relationship between dose-rate and the occur-
rence of interstitial pneumonitis was not observed in the
208 patients who received cyclosporine.

The relationship between donor-recipient sex-match and
the occurrence of interstitial pneumonitis was similar to
that reported previously in a smaller series of patients (18),
and it approached statistical significance (p < 0.06). The
incidence was lowest among female patients who received
bone marrow from female donors (21%, 38 of 182) and
highest (34%, 77 of 227) in male patients who received
bone marrow from female donors. No other variable in-
vestigated (Table 2) was associated with the risk of inter-
stitial pneumonitis.

MULTIVARIATE ANALYSES

All six factors found to be significantly related to the
occurrence of interstitial pneumonitis in the univariate
analysis (Figure 1) were significant when simultaneously
included in the logistic equation. The transplantation
center was not a significant prognostic factor.

To simplify presentation of the results, we did a second
logistic analysis with dichotomized variables. The relative
risk of interstitial pneumonitis and the associated p val-
ues for the dichotomized variables are presented in Table
3.

When data from patients with idiopathic and cytomeg-
alomavirus-associated interstitial pneumonitis were tested
separately in logistic analyses, the only risk factor that
differed significantly (p < 0.04) was age of the patient.
The relative risk for older patients was 1.8 in those with
idiopathic disease and 3.2 in those with interstitial pneu-
onitis associated with cytomegalovirus. There was no
significant difference between small and large centers in
the incidence of interstitial pneumonitis or in the propor-
tion of patients with idiopathic as compared with cyto-
meagalovirus-associated disease.

Logistic analysis was used to estimate the probability of
developing interstitial pneumonitis for a patient with a
given profile of prognostic factors. The estimated proba-
bility was 8% when none of the adverse risk factors were
present, and this probability significantly increased as
each of the six significant adverse risk factors was added
(Figure 2). When all six adverse risk factors were pres-
ent, the probability of interstitial pneumonitis was 94%,
and the risk of a patient developing the disease was grea-
ter than 11 times higher than that seen in patients who
had none of the adverse risk factors.

The six factors of prognostic importance can be divid-
ed into two categories according to their ability to be

Of the 268 patients who developed interstitial pneu-
onitis, the disease was considered to be idiopathic in
50%; it was associated with cytomegalovirus in 37%,
with Aspergillus or Candida, with P. carinii in 4%, and
with herpes simplex virus, herpes zoster virus, Torulop-
sis, or Actinomycetes in 4%. The case-fatality rate was
78% (105 of 135) for patients with idiopathic disease,
93% (92 of 99) for those with cytomegalovirus, and
80% (28 of 35) for patients with other agents.

UNIVARIATE ANALYSES

To facilitate presentation of the data and for descript-
ive purposes, the results of the univariate analyses are
shown in Figure 1. The incidence of interstitial pneumo-
nitis was increased with increasing age of the patient
(Figure 1A), the use of methotrexate (Figure 1B), the

Figure 1A. Relationship between the patients' age at the time of
transplantation and the incidence of interstitial pneumonitis. Figure
1B. Incidence of interstitial pneumonitis among patients given cy-
closporine (CSA) or methotrexate (MTX) as prophylaxis against
graft-versus-host disease (GVHD). Figure 1C. Relationship between
the severity of acute graft-versus-host disease and the incidence of
interstitial pneumonitis. Figure 1D. Relationship between the
performance rating before transplantation (Pre-Tx) and the
incidence of interstitial pneumonitis. Figure 1E. Relationship
between the interval from diagnosis to transplantation and the
incidence of interstitial pneumonitis. Figure 1F. Relationship
between dose-rate of irradiation and incidence of interstitial
pneumonitis in patients who were treated after transplantation with
methotrexate to prevent graft-versus-host disease. The number
within each bar indicates the number of patients in the group, and the
p values are derived by univariate analyses.

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Table 3. Significant Risk Factors for Interstitial Pneumonitis Identified from Multiple Logistic Regression Analyses

<table>
<thead>
<tr>
<th>Prognostic Variable</th>
<th>Lower Risk</th>
<th>Higher Risk</th>
<th>Relative Risk</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug to prevent graft-versus-host disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of patient</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity of graft-versus-host disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interval from diagnosis to transplantation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Performance rating before transplantation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose-rate of total body irradiation</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Controlled by the referring physician or transplantation team. Controllable variables include the drug used to prevent graft-versus-host disease, interval from diagnosis to transplantation, and dose-rate of total body irradiation. Uncontrollable variables include age of the patient, performance rating before transplantation, and severity of graft-versus-host disease. When all three controllable risk factors were unfavorable, the relative risk of interstitial pneumonitis was 4.5. When the three uncontrollable factors were unfavorable, the relative risk was 6.1.

ROLE OF RADIATION THERAPY REGIMEN

In addition to dose-rate (Figure 1F, Table 3), several irradiation variables were tested for possible associations with interstitial pneumonitis. The incidence of interstitial pneumonitis for patients exposed to x-irradiation was similar to that for patients given gamma-irradiation (30%, 169 of 564 compared with 28%, 97 of 352). The rate was somewhat higher (p < 0.08) in patients given lung doses of 12 Gy in five or six fractions (36%, 25 of 70) than in patients given 10 Gy in one fraction (24%, 29 of 121). These doses were selected for comparison because data from animal experiments indicate that 10 Gy in a single dose has a radiobiological effect that is equal to or greater than that of 12 Gy of fractionated irradiation (28, 29). Thus, our data do not indicate that fractionation, at least in the doses and schedules used, provides protection against interstitial pneumonitis. No dose-response relationship was found between the occurrence of interstitial pneumonitis and mean lung doses of irradiation within the range of 5.6 to 12.5 Gy (Figure 3). This relationship was still not significant after we adjusted for dose-rate and number of fractions by using logistic analysis.

Discussion

Our analysis of 932 patients with leukemia who received bone marrow transplants from HLA-identical siblings demonstrates several important risk factors associated with the development of interstitial pneumonitis.

DRUG USED TO PREVENT GRAFT-VERSUS-HOST DISEASE

As in a previous report by the International Bone Marrow Transplant Registry in a smaller study (18), we again found a strong association between the use of methotrexate and an increased risk of interstitial pneumonitis. Both cyclosporine and methotrexate are immunosuppressive and may predispose to infection from endogenous or exogenous organisms. Methotrexate also, however, has been reported to be associated with pulmonary toxicity via several mechanisms: direct toxicity, changes in number and function of pulmonary alveolar macrophages and neutrophils, and allergy (30-32). Particularly relevant are reports describing interactions between methotrexate and radiation and between methotrexate and cyclophosphamide resulting in additive and synergistic pulmonary toxicity (33-35). Moreover, methotrexate usually is administered after transplantation at frequent intervals, and this frequent dosing, rather than cumulative dose, correlates with lung damage (36). Thus far, most data from animal or human studies (37, 38) have suggested no direct toxicity of cyclosporine to the lung.
One recent study supports our finding that methotrexate given after transplantation is associated with an increased risk of interstitial pneumonitis (39). Patients who received methotrexate showed an increased incidence of interstitial pneumonitis (44%, 15 of 34) in comparison with the group that received no treatment to prevent graft-versus-host disease (14%, 3 of 21). This difference was unrelated to the relative incidences of graft-versus-host disease in the two groups.

In contrast to our study, a randomized clinical trial comparing methotrexate and cyclosporine has failed to indicate a significant difference in the incidence of interstitial pneumonitis: 22% (9 of 41) and 25% (9 of 36), respectively (40). The reason for this disparity is unclear. The relatively small number of patients in that trial and resultant large beta-error made the likelihood of detecting a significant difference less than 50%.

**AGE OF PATIENT**

Younger patients had a lower incidence of interstitial pneumonitis compared with older patients, confirming a similar observation made at one center (17). The reason for this difference is unclear but could be related to differences in previous lung damage or to age-related differences in drug metabolism, toxicity, tissue resistance to radiation or drugs, or ability to repair tissue injury. The difference might also reflect the increased likelihood of older patients having had prior exposure to cytomegalovirus (with reactivation after transplantation). This suggestion is supported by the increasing rate of seropositivity to cytomegalovirus with increasing patient age (41). This possibility is supported further by our observation that cytomegalovirus pneumonia occurred in only 6% (27 of 451) of patients less than 21 years of age, whereas the incidence was 15% (72 of 478) in older patients. Although age of the patient was an important risk factor for idiopathic interstitial pneumonitis, the association was significantly stronger among patients with cytomegalovirus-associated disease.

**SEVERITY OF GRAFT-VERSUS-HOST DISEASE**

A strong association was found between the severity of graft-versus-host disease and the incidence of interstitial pneumonitis. Similar (17, 23, 25) and dissimilar (22, 42) results have been reported. Both graft-versus-host disease and its treatment result in profound and prolonged immune suppression after transplantation (14, 15), which may contribute to the increased risk of interstitial pneumonitis. Furthermore, graft-versus-host disease and its treatment can activate latent cytomegalovirus infection in mice (43). Activation of cytomegalovirus can affect the speed and extent of immune reconstitution (44). Patients in our study who developed moderate to severe acute graft-versus-host disease had a relative risk of interstitial pneumonitis associated with cytomegalovirus of 2.5 compared with a relative risk of 1.6 for those with idiopathic interstitial pneumonitis. Similar results were found in a retrospective study of cases not included in this report (19).

**INTERVAL FROM DIAGNOSIS TO TRANSPLANTATION**

The reason for an association between interval from diagnosis to transplantation and the risk of interstitial pneumonitis is uncertain. Patients who receive transplants at a longer interval from diagnosis are exposed to an increased duration and total dose of chemotherapy, which may lead to greater subclinical pulmonary injury and a greater degree of immunoincompetence. The increased incidence of interstitial pneumonitis among patients who received transplants more than 6 months after diagnosis was not due to an increased number of transfusions before transplantation with an associated higher risk of transfusion-transmitted cytomegalovirus infection. Patients who were given transplants within 6 months of diagnosis and those given transplants at longer intervals received similar numbers of transfusions before transplantation (mean, 24.8 ± 25.8 and 21.4 ± 26.2, respectively).
RADIATION THERAPY REGIMEN

Dose-Rate of Irradiation: Radiation to the lung can cause pulmonary damage (45). In this analysis we confirm our previously reported correlation between higher dose-rates and an increased risk of interstitial pneumonitis (18). The correlation was significant, however, only for the 672 patients who received methotrexate after transplantation. This finding is in accord with reports indicating that lung damage is increased when irradiation and methotrexate therapies are combined (33-35). Dose-rates of irradiation did not influence risk of interstitial pneumonitis in patients who received cyclosporine. The relationship between dose-rate of irradiation and occurrence of interstitial pneumonitis is probably complex. Animal studies have suggested that the relationship should be affected by total lung dose and fractionation schedules (28, 29, 46). However, we found that the relationship did not depend on these factors, but rather depended on a combined effect of dose-rate and methotrexate.

Dose of Irradiation: Within the range of 5.6 to 12.8 Gy we found no dose-response effect of pulmonary irradiation. Adjusting for number of fractions and dose-rate did not alter this conclusion. Similarly, Sloane and colleagues (20) have found no dose-response relationship between lung doses of 9.1 to 13.0 Gy and the incidence of interstitial pneumonitis. This finding contrasts with those of Keane and colleagues (43), who reported a direct correlation between the dose of irradiation to the lung and the risk of interstitial pneumonitis. The measurement of the actual dose delivered to the lungs, however, is difficult (46). It is possible that inaccuracies in dose measurement and differences in methods of reporting between centers may have obscured a dose-response relationship. On the other hand, the range of doses used in preparing patients with leukemia for transplantation may have been below or above the level where a dose-response relationship would be detectable clinically.

Fractionated Irradiation: We found no evidence that fractionated irradiation reduced the risk of interstitial pneumonitis. Similar findings have been reported in randomized and nonrandomized trials (47, 48). Other investigators, in small uncontrolled studies, have reported a decreased incidence of interstitial pneumonitis when fractionated irradiation was used (23, 49, 50), but these results must be interpreted cautiously because of the high incidence (58% to 70%) of interstitial pneumonitis in their control patients who were given single-dose irradiation. In two of the studies (23, 49), although the incidence of interstitial pneumonitis was reduced in patients given fractionated irradiation, the rate still exceeded the 29% overall rate in our study. Thus, within the range of doses and schedules tested, fractionated irradiation did not prove useful in decreasing the risk of interstitial pneumonitis.

PERFORMANCE STATUS BEFORE TRANSPLANT

The reason for the reduced risk of interstitial pneumonitis among patients with 100% performance ratings before transplantation compared with patients with ratings of 90% or less is not known. The difference may reflect greater tissue integrity with superior resistance to lung damage by the preparatory regimen for patients who were able to carry on normal activities with no evidence of disease, in comparison with patients whose performance status was even minimally compromised before transplantation. The relationship between performance status and incidence of interstitial pneumonitis was dichotomous. Patients with a 100% rating had an incidence of 24%, compared with 39% in those whose rating was less than 100%. In the group with the poorer ratings, there was no significant trend over the range of 20% to 90% (Figure 1D). It is difficult to assign precise performance scores. A rating of less than 100% may have reflected a clinical impression that although the criteria for a 100% rating were present, the patient was not an ideal candidate for transplantation. Therefore, we believe that conclusions regarding the role of the performance rating before transplantation on the risk of interstitial pneumonitis should be viewed with caution.

RELIABILITY OF DATA

One major concern regarding the results of this study relates to the contribution of transplantation centers of varied size and expertise. In no instance could we detect a "center-effect"; factors predictive of interstitial pneumonitis were valid at small as well as large centers, and data from no single center unduly influenced the study conclusions. A second related concern was the accurate distinction between idiopathic interstitial pneumonitis and that associated with cytomegalovirus and other organisms. Differences in diagnostic expertise undoubtedly exist between centers, but our concern for this issue was lessened because in more than 75% of the cases the diagnosis was established with biopsy or autopsy material. It is therefore unlikely that differences in diagnostic expertise lessened the validity of our conclusions.

Data from this study suggest that multiple factors predict, and probably contribute to, interstitial pneumonitis that occurs after bone marrow transplantation. We emphasize that these analyses were not based on randomized clinical trials. Clinical techniques, experience, and number of patients varied widely among the 69 centers that provided data for this study. Data from any registry have inherent weaknesses associated with inconsistent recording of information and unrecorded factors that confound treatment choices. These problems have been reduced in data reported to the International Bone Marrow Transplant Registry through the use of protocols for uniform reporting of data, through careful and comprehensive recording of patient and treatment information, and through the statistical techniques used. A major advantage of this registry, compared with clinical trials, is that it has a sufficiently large sample size and variability of a number of important prognostic variables that would be difficult or impossible to investigate at a single center. The results of the study reported here can be used to estimate the risk of interstitial pneumonitis for an individual patient and to suggest controlled clinical trials to test specific selection criteria and treatments.
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References


Circulating Coagulation Inhibitors in the Acquired Immunodeficiency Syndrome

ALICE J. COHEN, M.D.; TERRENCE M. PHILIPS, Ph.D.; and CRAIG M. KESSLER, M.D.;
Washington, D.C.

Abnormal coagulation profiles were identified in ten patients with the acquired immunodeficiency syndrome (AIDS) associated with opportunistic infections and malignancies. Partial thromboplastin times were elevated in all patients; three of seven had elevated prothrombin times. All patients had lupus-type anticoagulants characterized by rapid prolongations of the partial thromboplastin time in mixing studies, prolonged dilute thromboplastin inhibition assays, and increased Russell viper venom clotting times. Iby bleeding times were prolonged in three patients with defective platelet aggregation. The lupus anticoagulant was isolated from the sera of healthy heterosexual men and from patients with AIDS with and without the lupus anticoagulant, and in the presence and absence of opportunistic infections. Both polyclonal IgM and IgG from plasma with lupus anticoagulant interfered with clotting studies and platelet aggregation. The inhibitors usually suppressed damped active opportunistic infections and tended to disappear with successful resolution of infection.

The lupus-type circulating anticoagulant was initially detected and characterized in patients with systemic lupus erythematosus (1-3); however, similar inhibitors also have been acquired in association with various other chronic autoimmune diseases, hematologic and nonhematologic malignancies, acute drug hypersensitivity reactions, and in pregnancy (2-6). Occasionally, the lupus anticoagulant may occur as an isolated idiopathic phenomenon (5, 6). The lupus anticoagulants appear to be abnormal acquired IgG or IgM immunoglobulins, or both, which are directed against phospholipid components of the prothrombin activator complex involved in coagulation (7-9) and occasionally against the phospholipid contained in the platelet membrane (10, 11). The resulting inhibition of coagulation and platelet function can be shown in vitro (4, 10, 12), but rarely produces clinical hemorrhagic complications (2, 4, 6) unless the lupus anticoagulant is associated with a qualitative or quantitative platelet defect, or an absolute prothrombin deficiency (2, 4, 6, 8, 10, 11, 13).

Recently, we have seen a circulating anticoagulant in patients with the acquired immunodeficiency syndrome (AIDS). We have shown that these inhibitors of coagulation associated with AIDS may be monoclonal or polyclonal immunoglobulins, that they may produce qualitative platelet defects, and that they appear to arise and subside spontaneously with the onset and successful resolution of opportunistic infections. The in-vitro properties of the agents would allow them to be designated lupus anticoagulants.

Methods

PATIENT POPULATION

Fifty homosexual male patients fulfilling the Centers for Disease Control (CDC) criteria (14) for the diagnosis of AIDS.

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HLA Associations With Leukemia

By Mortimer M. Bortin, Joseph D’Amaro, Fritz H. Bach, Alfred A. Rimm, and Jon J. van Rood

Frequencies of 35 HLA A, B, C, and DR antigens were determined in 1,834 leukemic Caucasoids to evaluate possible associations between HLA and leukemia. In comparison with the frequencies of HLA antigens in published controls, the frequency of CW3 was significantly higher in patients with acute lymphoblastic leukemia (relative risk = 2.64, P < 0.0002), acute myelogenous leukemia (relative risk = 1.92, P < 0.0007), and chronic myelogenous leukemia (relative risk = 2.07, P < 0.0002; values adjusted for multiple comparisons). The frequency of CW4 was elevated in patients with acute lymphoblastic leukemia (relative risk = 2.01, P < 0.0003), acute myelogenous leukemia (relative risk = 2.06, P < 0.0002), and chronic myelogenous leukemia (relative risk = 2.14, P < 0.0008). The frequency of AW19 was significantly decreased in patients with acute myelogenous leukemia (relative risk = 0.68, P < 0.01) and chronic myelogenous leukemia (relative risk = 0.59, P < 0.006). None of the other 32 HLA antigens investigated had a statistically significant association with leukemia. The data suggest that CW3 and CW4 may be markers for leukemia susceptibility genes, while AW19 may be a marker for decreased susceptibility to leukemia.

In 1930 Fisher proposed that selective interactions between genes might favor their linkage. Darlington and Mather extended this idea and introduced the term “supergene” for groups of linked genes that are inherited en bloc. Overviews of the HLA supergene were published by Ceppellini, Bach and van Rood, Bodmer, and Bach. The biological importance of supergenes is emphasized by the fact that they are found in all vertebrate species that have been studied. Homology between the major histocompatibility complex (MHC) in different species supports the hypothesis that the MHC has been conserved throughout phylogeny because it confers important biological advantages on the species.

The observation by Lilly et al. that increased or decreased susceptibility of mice to Gross virus-induced leukemia was linked to H-2 and H-2 respectively prompted Amiel in 1967 to search for associations between histocompatibility antigens and pathologic conditions in humans. He reported a significant association for HLA B5 in patients with Hodgkin’s disease. Since then numerous studies investigated HLA associations with disease.

In 1984 we reported the results of analyses investigating HLA associations with leukemia in 1,009 leukemia patients (hereafter referred to as “initial series”). We found a statistically significant increase in the frequency of CW3 and CW4 in patients with acute lymphoblastic leukemia (ALL) and of CW4 in patients with acute myelogenous leukemia (AML). An additional 825 leukemia patients have now been studied (“current series”), and the results from these patients and the combined series are presented here.

PATIENTS AND METHODS

Patients. Detailed information regarding 1,834 North American and European Caucasoid patients treated with chemotherapy and bone marrow transplantation (BMT) for leukemia was reported to the International Bone Marrow Transplant Registry. Transplants were performed between 1969 and 1985 by 96 BMT teams worldwide. The diseases studied were ALL, AML, and chronic myelogenous leukemia (CML; Table 1). Patients’ median age was 21.4 years (range 0.6 to 59 years), and 55% were males. The median interval from diagnosis to transplantation was 9 months (range 0.3 to 144 months). At the time of transplantation 47% of the patients were in first remission or chronic phase, 20% in second remission, and the disease was more advanced in 33%. Chemotherapy failed to induce a first remission in 3% of the patients with acute leukemia.

Antigenic splits were converted to their equivalent broad specificities because many of the splits were not defined in the earlier years of this study. Data for 35 HLA antigens were available for investigation: eight HLA A antigens (1,2,3,9,10,11,w9,28), 15 HLA B antigens (5,7,8,12,13,14,15,16,17,18,21,w22,27,35,46), six HLA C antigens (w1,w2,w3,w4,w5,w6), and six HLA DR antigens (1,2,3,4,5,7). Estimates of the frequency of DRw6 in North American and European Caucasian controls were not thought to be reliable, and this antigen was excluded from analysis. Data were available to analyze the frequency of HLA A in 1,834 patients, B in 1,801 patients, C in 783 patients, and DR in 709 patients.

HLA antigen frequencies for the control series of North American and European Caucasoids were obtained from Tables 6 through 9 in Baer and Daniloff’s population analysis from the Eighth International Histocompatibility Workshop.

Statistical analysis. The extensive polymorphism of HLA not only provides a large number of markers to be tested for possible associations with diseases but also presents problems in interpretation of results. The main problem is that the performance of large numbers of statistical comparisons increases the probability that apparent differences between two groups may have occurred solely by chance. Svejgaard et al. discussed this problem and suggested several methods to help resolve it.

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Table 1. Distribution of Patients by Disease

<table>
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<th>Disease</th>
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<th>Current Series</th>
<th>Total</th>
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<td>ALL</td>
<td>423</td>
<td>304</td>
<td>727</td>
</tr>
<tr>
<td>AML</td>
<td>383</td>
<td>282</td>
<td>665</td>
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<tr>
<td>CML</td>
<td>203</td>
<td>239</td>
<td>442</td>
</tr>
<tr>
<td>Total</td>
<td>1,009</td>
<td>825</td>
<td>1,834</td>
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</table>

Haldane’s modification10 of Woolf’s method29 was used to assess the significance of the differences in the antigen distributions in the control vs the leukemia populations. Multiplex testing for heterogeneity in a single locus or multiple loci was performed by summing the chi squares provided by the Woolf-Haldane analysis. That value is itself a chi square with degrees of freedom equal to the total number of antigens in the loci or loci.29 Corrected P values were derived by taking into account the 35 antigens that were tested using the method of Edwards.29 Only corrected P values are presented.

Initially HLA antigen frequencies from the 258 North American and the 567 European Caucasoids from the current series were tested independently against their respective controls. Comparisons were then made with the HLA antigen frequencies found in the 628 North American and 381 European Caucasoids from the initial series. This strategy was used because it would be most unlikely that the same association would be observed by chance alone in all four subsets. All four subsets were combined for an overall analysis.

RESULTS

Multiplex testing disclosed no significant heterogeneity in the distribution of HLA A, B, C, and DR antigens between the North American and Eurcjenan patients in the initial or current series. In particular there were no significant differences for the Aw19, Cw3, and Cw4 antigens. The distribution of the Aw19, Cw3, and Cw4 antigens in the North American and European control populations also did not differ significantly.

Of the 35 HLA antigens studied, only Aw19, Cw3, and Cw4 had significant associations with leukemia in general, ie, the combined series ALL, AML, and CML (Table 2). Compared with controls, Aw19 had a lower frequency and decreased relative risk in all four subsets of patients; the pooled data had a frequency of 23% vs 30% for controls with a relative risk of 0.71 and a P value < 0.0006. Both Cw3 and Cw4 had consistently elevated frequencies in all four subsets in comparison with controls, and the relative risks of 2.20 and 2.06 respectively for the pooled data were highly significant (P < 0.00001).

Shown in Table 3 are the relative risks and significance levels for Aw19, Cw3, and Cw4 in the initial, current, and combined series for ALL, AML, and CML. For the Aw19 antigen, note that the relative risk was consistently below 1.0 in each of the North American and European subsets and for each of the diseases studied, but in none of the 12 subsets was statistical significance observed. It was not until the data were pooled, with a resultant increase in the power of the test, that statistically significant lower frequencies for Aw19 were found both for the 665 patients with AML and the 442 patients with CML. In the pooled data from 727 patients with ALL, the frequency of Aw19 was lower than controls, but the level was not statistically significant.

The frequency for Cw3 was consistently elevated for patients with ALL, AML, and CML but was statistically significant in only three of four ALL, in one of four AML, and in one of four CML subsets (Table 3). When the data were pooled, the power of the test increased, and the frequency of Cw3 was significantly increased for each of the diseases. Similarly, the frequency of Cw4 was consistently elevated for all 12 subsets of patients but was statistically significant in only two subsets of AML patients. The combined data indicate that Cw4 has a significantly increased frequency for ALL, AML, and CML.

In the combined series there were 72 patients with ALL or AML in whom complete remission was not attained following induction chemotherapy. They were considered to be refractory to chemotherapy. To determine whether Aw19, Cw3, or Cw4 might be associated with responsiveness to chemotherapy in leukemia patients rather than leukemia per se, the frequencies of these antigens were analyzed in the 1,320 patients with acute leukemia who had responded to chemotherapy and the 72 patients who had not. No statistically significant heterogeneity was found; chi squares were 1.08 for Aw19; 0.03 for Cw3; and 0.01 for Cw4. Among the patients who had not responded to chemotherapy, the frequency of Aw19 was not significantly different from patients who had responded: 31% vs 30% respectively. The frequency of Cw3 of nonresponders, however, was increased; 36% vs 20% for controls (uncorrected P = 0.012), as was the frequency of Cw4; 40% vs 22% for controls (uncorrected P = 0.005).

DISCUSSION

This investigation of 1,834 patients with leukemia disclosed highly significant associations between HLA and the risk of leukemia. In comparison with healthy controls, frequencies of Cw3 and Cw4 were increased (relative risk > 2.0, P < 0.00001), and the frequency of Aw19 was decreased

Table 2. Woolf-Haldane Analysis of HLA Antigens Significantly Associated with ALL, AML, and CML

<table>
<thead>
<tr>
<th>Antigen</th>
<th>C (%)</th>
<th>North American</th>
<th>Current Series</th>
<th>Combined Series</th>
<th>European</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L (%)</td>
<td>RR</td>
<td>L (%)</td>
<td>RR</td>
</tr>
<tr>
<td>Aw19</td>
<td>30</td>
<td>0.68</td>
<td>24</td>
<td>0.75</td>
<td>24</td>
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<tr>
<td>Cw3</td>
<td>20</td>
<td>2.01</td>
<td>34</td>
<td>2.18</td>
<td>34</td>
</tr>
<tr>
<td>Cw4</td>
<td>22</td>
<td>2.06</td>
<td>41</td>
<td>2.35</td>
<td>39</td>
</tr>
</tbody>
</table>

*Antigen frequencies for North American and European controls.29
†Antigen frequencies for patients with leukemia.
"Relative risk.
§Corrected for 35 comparisons.
### Table 3. Woolf-Haldane Analysis of Aw19, Cw3, and Cw4 Associations with Leukemia

| Antigen | Disease | Initial Series | | | Current Series | | | | Combined Series | | |
|---------|---------|---------------|---|---|---------------|---|---|---|---------------|---|
| Aw19    | ALL     | 0.84        | NS | 0.88 | NS          | 0.64 | NS | 0.93 | NS | 0.84 | NS | |
| Aw19    | AML     | 0.62        | NS | 0.67 | NS          | 0.51 | NS | 0.64 | NS | 0.66 | <0.01 |
| Aw19    | CML     | 0.49        | NS | 0.68 | NS          | 0.83 | NS | 0.56 | NS | 0.59 | <0.006 |
| Cw3     | ALL     | 2.54        | .002 | 3.19 | .002 | 1.33 | NS | 2.84 | .003 | 2.64 | <0.0002 |
| Cw3     | AML     | 1.34        | NS | 2.00 | NS          | 2.22 | NS | 2.25 | .03 | 1.92 | <0.0007 |
| Cw3     | CML     | 3.44        | .01 | 1.14 | NS          | 2.37 | NS | 1.78 | NS | 2.07 | <0.002 |
| Cw4     | ALL     | 2.01        | NS | 2.26 | NS          | 2.58 | NS | 1.82 | NS | 2.01 | <0.0003 |
| Cw4     | AML     | 2.46        | .006 | 2.52 | .018 | 1.78 | NS | 1.72 | NS | 2.06 | <0.0002 |
| Cw4     | CML     | 1.41        | NS | 2.29 | NS          | 3.59 | NS | 2.08 | NS | 2.14 | <0.0008 |

*Relative risk.  
†Corrected for 35 comparisons.

(relative risk <0.72, P < 0.0006). These findings confirm and extend our previous report investigating HLA association with leukemia.\[16\] Frequencies for the 32 other A, B, C, and DR antigens studied were not significantly different from controls.

Most studies of HLA associations with leukemia have focused on the HLA A, B, or D/DR loci.\[13,14\] The findings have been inconsistent, and no strong associations between these loci and leukemia have been reported (Table 4). In particular, to the best of our knowledge, associations between HLA C and leukemia were not reported prior to or since our initial study.\[16\] De Jongh et al.\[26\] conducted the only study known to us in which HLA C associations with leukemia were investigated. They found no significant difference in HLA C antigen frequency between controls and a group of 44 children with ALL. The discrepancy between their results and ours may have been due to the low power of the test in their study.

The pooled data showed that Aw19 had a significantly lower frequency among leukemia patients in general. In our initial study the lower frequency of Aw19 in leukemia patients was uncertain because it had only borderline significance (corrected P = 0.047) in the North American subset of 628 patients and was not significant in the 381 European patients.\[16\] With the group size increased from 1,009 to 1,834 leukemia patients, it now seems clear that the frequency of Aw19 is indeed decreased in leukemia patients. The frequency of Aw19 was significantly reduced in AML (P < 0.01) and CML (P < 0.005). Although not statistically significant in ALL, it should be noted that the relative risk associated with Aw19 was consistently lower than 1.0 in all four subsets of ALL patients (Table 3). In one study of 60 patients with ALL, a decreased frequency of Aw19 (uncorrected P < 0.05) was reported by Kluoda et al.\[31\] It is possible that when additional patients with ALL become available for study, Aw19 may also prove to be associated with a reduced risk for this disease.

The strong positive association of Cw3 and Cw4 with leukemia supports the possibility that these antigens, or the genes encoding them, are, or are closely linked with, possible susceptibility genes. An increased relative risk of leukemia has been observed in the siblings of leukemia patients,\[4,11\] but the mechanism is not known. Based on the findings reported here, it is possible that the mechanism may be due in part to an increased frequency of Cw3 or Cw4 in siblings of leukemia patients. Studies of HLA C in instances of familial leukemia are encouraged. Another possibility, suggested by other studies, is that Aw19, Cw3, and Cw4 may be markers coding for immune responsiveness to putative leukemia viruses; DeVries et al.\[16\] found a significant association between Cw3 and low in vitro responses to vaccinia virus. Our data show no association between Aw19, Cw3, or Cw4 and responsiveness to chemotherapy in leukemia patients.

The reader is cautioned that our findings may not be representative of all leukemia patients because of several unavoidable biases in the database of the International Bone Marrow Transplant Registry. For example, most teams do not perform BMTs on older patients. As a consequence, almost all patients in this study were less than 50 years of age, and our findings may not apply to older leukemia patients. The 95% remission-induction rate in our cases with acute leukemia is higher than most reports of nontransplanted patients, especially those with AML. All patients reported here survived long enough to undergo BMT. It is likely that some patients for whom BMT was planned died before the plan could be implemented. It is possible that patients with especially virulent forms of leukemia or, as suggested by Rogentine et al., some less hardy patients did not survive long enough to be transplanted and thus were not available to us for study. Other interpretations are possible. Furthermore, HLA data for patients with chronic lymphocytic leukemia were not available to us for analysis, and it is not known whether the findings reported here also apply to these patients or to non-Caucasoids. Studies of HLA associations with morphological, immunologic, and cytogenetic subtypes of leukemia will be undertaken in the future with an expanded database.

The positive and negative associations with leukemia we found with relative risks ranging from 2.64 to 0.59, while highly significant, are weak in comparison with those described in some other studies showing significant associations between HLA and disease.\[15,15\] The extremes, according to Svejgaard et al.,\[14\] are the positive association between B27 and ankylosing spondylitis, with a relative risk of 87.4, and the negative association between DR2 and insulin-dependent diabetes, with a relative risk of 0.2. The analyses presented...
Table 4. Summary of Representative Reports in Which Associations Between HLA and Leukemia Were Investigated

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. Patients</th>
<th>No. Antigens Studied</th>
<th>Significant Antigen</th>
<th>Frequency*</th>
<th>P</th>
<th>Corrected†</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
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<td>28</td>
<td>27 A, B</td>
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<td>—</td>
<td>—</td>
<td>23</td>
</tr>
<tr>
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<td>—</td>
<td>—</td>
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<td>—</td>
<td>25</td>
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<td>—</td>
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<td>&lt;.001</td>
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<tr>
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<td>31</td>
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<tr>
<td>AML</td>
<td>665</td>
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<td>Cw4</td>
<td>Increased</td>
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<td>31</td>
</tr>
<tr>
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<td>Cw4</td>
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<td>&lt;.0001</td>
<td>Yes</td>
<td>31</td>
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<td>—</td>
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<td>&lt;.02</td>
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<td>A3</td>
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<td>Cw4</td>
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<td>B21</td>
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<td>35 A, B, C, DR</td>
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<td>Increased</td>
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<td>35 A, B, C, DR</td>
<td>Cw4</td>
<td>Increased</td>
<td>&lt;.0001</td>
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<td>35 A, B, C, DR</td>
<td>Cw4</td>
<td>Increased</td>
<td>&lt;.0001</td>
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<td>31</td>
</tr>
</tbody>
</table>

*Compared with healthy controls.
†P value corrected for multiple comparisons.
‡This report.

Here clearly show the desirability of a large database in studies investigating HLA associations with disease when there is in fact a relationship but it is not strong. For example, in Table 3, although a trend was present, Aw19 was not significantly associated with AML or CML in any of the subsets. Only when group sizes in the combined series were 665 (AML) and 442 (CML) did statistically significant associations become evident. Similarly, Cw4 was not statistically significant in any of the ALL or CML subsets; using pooled data from the combined series demonstrated highly significant associations with each disease. Another problem with relatively small group sizes is demonstrated by those
REFERENCES

instances when significant associations were found in some subsets but not in others (Table 3). It is clear from the data shown in Table 4 that inadequate group sizes account for many of the conflicting reports in the literature describing the presence or absence of HLA associations with leukemia.

The increased frequency of CW3 and CW4 and decreased frequency of Aw19 in leukemia patients provides strong evidence that the HLA supergene, as H-2 in the mouse, may play a role in increased and decreased susceptibility to leukemia. Although it is far from clear that the mechanism for the association in mouse and man is similar, until now the parallel has been elusive.

It is generally believed that HLA antigens influence the T lymphocyte repertoire. We found that the risk of leukemia is significantly increased in individuals with CW3 or CW4. It seems likely that these people will have a T cell repertoire that is different from individuals who are negative for CW3 and CW4. This simply means that CW3 and CW4 antigens are immune response gene products. It does not answer the question of how these immune response genes confer low responsiveness against a putative leukemia-inducing agent. Our data imply that individuals who have neither CW3 nor CW4 are better able to resist leukemia than those who are positive. This remains purely speculative because there are no available tests to investigate this hypothesis.

The fundamental importance of this study is not that CW3 and CW4 were found to have significant associations with leukemia but rather that HLA C genes, or genes closely linked with HLA C, have hitherto unrecognized properties. Whether HLA C also will be found to be associated with other malignant and nonmalignant diseases remains to be determined.

ACKNOWLEDGMENT

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APPENDIX

This 32nd report from the International Bone Marrow Transplant Registry was prepared for the Advisory Committee: R.P. Gale, Los Angeles (Chairman); F.H. Bach, Minneapolis; A.J. Barrett, London; D.W. van Bekkum, Rijswijk; J.C. Biggs, Sydney; K.G. Blume, Los Angeles; M.M. Bortin, Milwaukee; K.A. Dicke, Houston; E. Gluckman, Paris; J.M. Goldman, London; R.A. Good, St. Petersburg; R. Hong, Madison; R.H. Herzog, Cleveland; J.H. Kersey, Minneapolis; H.J. Kolb, Munich; A.M. Marmont, Genoa; T. Masataka, Osaka; H.A. Messner, Toronto; R.J. O'Reilly, New York; R.L. Powles, London; A.A. Rimm, Milwaukee; O. Ringden, Huddinge; J.J. van Rood, C. Rozman, Barcelona; B. Speck, Basel; B.S. Weiner, Gainesville; and F.E. Zwaan, Leiden.

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Temporal relationships between the major complications of bone marrow transplantation for leukemia

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Summary:

Data from 3113 patients receiving HLA-identical sibling bone marrow transplants for leukemia were analysed to determine the time course of the major causes of treatment failure. The median interval from transplant to onset of acute graft-versus-host disease (GVHD) was 17 days, interstitial pneumonia 63 days, and chronic GVHD 111 days. The median interval from transplant to relapse was 3.3 months for patients transplanted in relapse of acute leukemia or blast phase of chronic myelogenous leukemia (CML), 6.4 months when transplants were performed in second or subsequent remission of acute leukemia or accelerated phase of CML, and 7.8 months for patients transplanted during first remission of acute leukemia or while in the chronic phase of CML. Shorter intervals from transplant to onset of interstitial pneumonitis or chronic GVHD were associated with a significantly lower probability of 2-year survival. The temporal relationships between these complications are displayed graphically and demonstrate the overlapping and competing causes of death following allogeneic bone marrow transplantation.

The first year following allogeneic bone marrow transplantation (BMT) for leukemia is the period of greatest risk for treatment failure (death or relapse). Patients who survive this critical interval have an excellent chance for long-term, leukemia-free survival.

The major causes of failure after allogeneic BMT for leukemia are acute graft-versus-host disease (GVHD),

interstitial pneumonitis (IPn),

chronic GVHD and its associated complications, and relapse. The peak incidence of each of these complications occurs within the first year post-transplant. Interactions among them are complex and have a significant impact on outcome. For example, the risk and lethality of IPn is increased in the presence of GVHD, and the severity of GVHD may favorably or unfavorably affect the risk of leukemia relapse. The severity of GVHD correlates directly with the risk of infection and inversely with the probability of relapse.

Analyses were performed to investigate the sequence of events complicating HLA-identical sibling bone marrow transplants in leukemia. The findings help elucidate pathogenetic mechanisms involved in the major causes of treatment failure following BMT.

Patients and methods

Comprehensive data were reported to the International Bone Marrow Transplant Registry (IBMTR) by 131 transplant centers worldwide for 3113 patients with acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML). All patients received bone marrow transplants from HLA-identical siblings between 1 January, 1982 and 31 December, 1987. Their distribution by disease and disease status is shown in Table I. Patients transplanted in first remission of ALL or AML or the chronic phase of CML are referred to as having 'early' leukemia; patients in second remission of ALL or AML or the accelerated phase of CML as having 'intermediate' leukemia; and relapse of ALL or AML or blast phase of CML as having 'advanced' leukemia. Minimum follow-up time was 4 months, and the median was 19 months. Median patient age was 26.8 years (range 0.9–59 years) and 1789 (57%) were male. The pretransplant preparative regimen included cyclophosphamide in 2734 (88%) patients and total body radiation in 3011 (97%) patients. Prophylaxis against GVHD included methotrexate in 930 (30%) patients, cyclosporine in 1173 (38%), both methotrexate and cyclosporine in 328 (11%), T cell depletion in 600 (19%) and other methods in 82 (3%).

Events studied were acute GVHD, IPn, chronic GVHD and relapse. The overall incidence and actuarial probability of these complications are shown in Table II. To demonstrate the time when patients are at risk of each complication, intervals from transplant to onset and from transplant to death from the complication are plotted; the shorter intervals until the median are shown as the ascending limb of the curve and the intervals after the median as the descending limb. Standard life-table methods were used to estimate probabilities. The Lee–Desu statistic was used to test for significant differences between curves. The Kruskal–Wallis statistic was used to test for significant differences between medians.

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similar irrespective of the tumor burden present at the time of transplantation. The longer interval between transplant and relapse among patients who developed chronic GVHD suggests a possible graft-versus-leukemia effect. This putative adoptive immunotherapeutic effect was not apparent among patients developing acute GVHD.

In conclusion, most deaths occur in the first year following bone marrow transplantation for leukemia. The major causes of treatment failure are acute GVHD, IPH, chronic GVHD and relapse. Each has its own distinctive time-pattern with respect to the interval from transplant to onset and from onset to death. The time patterns overlap resulting in multiple competing causes of treatment failure.

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References

Occasional Survey

ALLOGENEIC BONE-MARROW TRANSPLANTATION FOR CHRONIC MYELOGENOUS LEUKAEMIA*

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Summary

In 117 patients with chronic myelogeneous leukaemia (CML) treatment with a combination of high-dose chemoradiotherapy plus transplantation of allogeneic bone marrow from HLA-identical, matched lymphocyte-culture-identical siblings resulted in an actuarial probability of 3-year survival of 63±16% (95% confidence interval) for 10 patients transplanted in chronic phase; 14±14% for 8 transplanted in accelerated phase; and 2±15% for 62 transplanted during blast crisis. Irrespective of disease status at the time of transplantation, and in contrast to chemotherapy, a plateau-effect was observed in the survival curves starting 14 to 19 months after transplantation.

INTRODUCTION

The treatment of chronic myelogeneous leukaemia (CML) has progressed little in the past 50 years. Median survival after chemotherapy is 3–4 years; fewer than 20% of patients survive 5 years, and there are no cures. There is no clear evidence that conventional chemotherapy can prolong life or delay the onset of blast crisis. After transition into blast crisis usually only partial and short remissions can be achieved with chemotherapy. Attempts to treat blast crisis with high-dose chemoradiotherapy followed by infusion of autologous stem cells harvested during chronic phase from bone marrow and blood have also given only short remissions. Bone marrow transplantation (BMT) in accelerated phase or blast crisis using healthy syngeneic or HLA-identical sibling donors has been disappointing owing to early infections, interstitial pneumonitis, failure to eradicate the leukaemia, and incomplete engraftment. Although cytotgenic conversion can usually be obtained even in patients allografted in blast crisis, long-term survival with the potential of cure has been reported in fewer than 20% of the patients.

Experience in patients with acute leukaemia clearly showed that the best results of allogeneic BMT were achieved during first or second complete remission. From these results several teams hypothesised that allogeneic BMT would have a better chance of success for patients with CML if the transplant was performed in first chronic phase rather than in later stages of the disease. In studies of patients with acute leukaemia treated with high-dose cyclophosphamide and total-body irradiation (TBI) morbidity and mortality were similar to those achieved with BMT. Most deaths were due to interstitial pneumonitis or graft-versus-host disease (GVHD). Although the observation period was relatively short, leukaemic relapse was less common in patients with CML than in patients with acute leukaemia treated with cyclophosphamide, TBI, and BMT.

The results of BMT in the chronic phase of CML from genetically identical twins are also encouraging. 8 of 12 patients reported in the literature remained in complete cytogenetic and haematological remission 21 to 60 months after transplantation.

PATIENTS AND METHODS

Data for 138 patients with CML transplanted between July 1, 1976, and June 30, 1982, were reported to the International Bone Marrow Transplant Registry (IBMTR) by 36 transplant centers. 117 of these patients received marrow from HLA-A, B, and mixed lymphocyte-culture (MLC)-identical allogeneic siblings with no other evidence of incompatibility. The minimum follow-up time was 13 months. 21 of the 138 patients did not meet the study criteria and were excluded from the analyses. Reasons for exclusions were: identical twin donor (8 patients), MLC technical failure or not done (6 patients), MLC incompatibility (3 patients), 1 patient each with HLA-A incompatibility, HLA-C incompatibility, or parental donor, and 1 patient with juvenile CML whose disease transformed into acute lymphoblastic leukaemia (ALL) before transplantation. The disease status at the time of transplantation for the 117 patients was evaluated just before the start of pretransplant...
Results

All but 7 patients were conditioned for transplantation with busulfan and TBI. 81 patients received additional antimetabolide drugs including L-asparaginase, busulfan, cytarabine, daunorubicin, dexamethasone, cyclophosphamide, methotrexate, thiotepa, and vinristine. Single-dose TBI was given to 61% of the patients; 15% received fractionated TBI, and 3 patients were not irradiated.

From preliminary study of the post-transplant survival curves (fig 1A), it was apparent that the patients could be divided into three major cohorts according to disease status of CML at the time of transplantation: 39 patients transplanted in first chronic phase, 56 patients transplanted in accelerated phase, and 22 patients transplanted in blast crisis. The distribution and frequency of 21 disease-related and transplant-related variables are presented in table 1.

Among the 39 patients transplanted in chronic phase, the actuarial probability of surviving 3 years was 6% (95% confidence interval CI 47-79%), the actuarial probability of 3-year survival for the 56 patients transplanted in accelerated phase was 36% (95% CI, 7-50%), and for the 22 patients transplanted in blast crisis the actuarial 3-year survival was 12% (95% CI, 0-27%). The difference in probability of survival for the three cohorts of patients was significant (p<0.001) after adjusting for other potentially important prognostic factors (fig 1A). The survival curves reached a plateau at 14, 19, and 17 months post-transplant for patients transplanted in chronic, accelerated, and blast phase, respectively.

Post-transplant relapse data for the three transplant groups are presented in fig 1B. The actuarial probability of relapse among patients transplanted in chronic phase was 7% in 59% CI, 0-16%) at 3 years. The probability of relapse for patients transplanted in accelerated phase (41%, 95% CI, 23-60%) and for patients transplanted in blast crisis (41%, 95% CI, 2-80%) was significantly higher (p<0.001) than for patients transplanted in chronic phase after adjusting for the variables shown in table 1.

3 of the 20 patients who relapsed were still alive at 8-7, 11-6, and 13-5 months after relapse. All were transplanted in accelerated phase, 1 is in chronic phase on chemotherapy, 2 received a second transplant, 1 of the latter relapsed again and is in chronic phase on chemotherapy 5 months after the second relapse, and the other is Ph-negative again 4 months after the second transplant.

### Table 1: Distribution and Frequency of Transplant-Cohort Variables According to Disease Status at Time of Transplantation

<table>
<thead>
<tr>
<th>Variable</th>
<th>1st chronic (n=39)</th>
<th>Combined (n=96)</th>
<th>Blast (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of patient (yr)</td>
<td>29.4 (19.4-42.2)</td>
<td>26.1 (11.6-61)</td>
<td>59.7 (21.5-82)</td>
</tr>
<tr>
<td>Age of donor (yr)</td>
<td>23.6 (14.0-36.1)</td>
<td>47.2 (21.0-80)</td>
<td>34.8 (20.0-55)</td>
</tr>
<tr>
<td>WBC at diagnosis (x 10^9/L)</td>
<td>101.9 (2.2-170)</td>
<td>110.9 (2.2-1000)</td>
<td>110.8 (2.2-1000)</td>
</tr>
<tr>
<td>Pre-transplant transfusions</td>
<td>14.0 (9.5-14.0)</td>
<td>5.0 (9.5-14.0)</td>
<td>8.0 (5.0-14.0)</td>
</tr>
<tr>
<td>Intervascular access to transplant (no)</td>
<td>21.4 (8.5-41.4)</td>
<td>21.4 (8.5-41.4)</td>
<td>21.4 (8.5-41.4)</td>
</tr>
<tr>
<td>Dose of total body irradiation (Gy)</td>
<td>10.0 (6.0-12.0)</td>
<td>10.0 (6.0-12.0)</td>
<td>10.0 (6.0-12.0)</td>
</tr>
<tr>
<td>Dose of marrow cells (x 10^9/kg)</td>
<td>4.1 (1.0-12.1)</td>
<td>4.1 (1.0-12.1)</td>
<td>4.1 (1.0-12.1)</td>
</tr>
<tr>
<td>Post-transplant intravascular (days)</td>
<td>27.4 (17.0-53.0)</td>
<td>27.4 (17.0-53.0)</td>
<td>27.4 (17.0-53.0)</td>
</tr>
<tr>
<td>Male patient</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
</tr>
<tr>
<td>Male donor, male patient</td>
<td>33.3 (32.5-34.1)</td>
<td>33.3 (32.5-34.1)</td>
<td>33.3 (32.5-34.1)</td>
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<tr>
<td>Male donor, female patient</td>
<td>33.3 (32.5-34.1)</td>
<td>33.3 (32.5-34.1)</td>
<td>33.3 (32.5-34.1)</td>
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<tr>
<td>Female donor, female patient</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
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<tr>
<td>Female donor, male patient</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
<td>66.7 (65.6-67.8)</td>
</tr>
<tr>
<td>Ph-negative</td>
<td>96.1 (95.6-96.6)</td>
<td>96.1 (95.6-96.6)</td>
<td>96.1 (95.6-96.6)</td>
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<tr>
<td>Previous splenectomy</td>
<td>80.6 (79.8-81.4)</td>
<td>80.6 (79.8-81.4)</td>
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<tr>
<td>Infected at transplant</td>
<td>10.0 (5.0-15.0)</td>
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<tr>
<td>Post-transplant chemotherapy</td>
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<tr>
<td>GVHD prophylaxis</td>
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<td>10.0 (5.0-15.0)</td>
<td>10.0 (5.0-15.0)</td>
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<tr>
<td>Cyclosporine</td>
<td>100.0 (100.0)</td>
<td>100.0 (100.0)</td>
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</tr>
</tbody>
</table>

*Median (range); †no. of patients in each transplant group.
experience for patients transplanted in accelerated phase was inferior to that of patients transplanted in chronic phase, their probability of survival was higher than for patients transplanted in blast crisis (fig 1A).

The actuarial risk of relapse among patients transplanted in chronic phase of CML was only 7% (fig 1B). Of the 2 patients who relapsed, 1 was Ph-negative and was given 200 mg/kg cyclophosphamide and 16 mg/kg busulphan but no TBI before transplantation. The other was Ph-positive and received 120 mg/kg cyclophosphamide, 1-88 mg/kg daunorubicin, and 10·0 Gy TBI before transplantation. This low relapse rate contrasts with data from the BHTR in which the risk of relapse following allogeneic BMT in first remission for acute myelogenous leukaemia (AML) was 32%10 and for ITLB 28%.17 The risk of relapse or persistent leukaemia in CML of 41% in both accelerated and blast phases (fig 1) also was somewhat lower than that observed in advanced AML (50%)4 and ALL (67%).17 The reason for the lower probability of relapse in CML than in AML and ALL is not known but may be due to differences in the virulence of the diseases or to greater vulnerability of CML malignant cells to high doses of TBI and cyclophosphamide than malignant cells in AML and ALL. Larger series of patients and longer follow-up are necessary to assess the longer term risk of relapse in CML.

Transplant-related mortality due to interstitial pneumonitis, GVHD, and infection remain as formidable problems in all grafted CML patients as in allografted AML and ALL patients. However, to determine the role of BMT in the management of patients with CML, the risks of severe transplant-related complications have to be compared with the risk of disease itself when other modes of therapy are used. With various chemotherapeutic agents, immunotherapy, splenectomy, the linearity of the decline in survival rates was inexorable and a plateau was not achieved.3,5-7,12 In contrast, a plateau effect was observed in the patients treated with BMT. The length of follow-up for the patients treated with BMT is, however, shorter than for the patients treated with conventional or experimental chemotherapy.

Thus, out of the 117 patients who died. Primary causes of death by disease status are shown in table I. Intestinal pneumonitis was the main cause of death, followed by recurrent or persistent leukaemia, infection, and GVHD.

Examination of the survival data for the 21 excluded patients disclosed that 5 of 8 patients (62%) who received marrow from identical twins are still alive 14-46 months post-transplant. 1 patients died of recurrent leukaemia. 1 of 6 patients who received marrow from HLA-identical donors but at whom the MLC tests were technical failures or not done are alive 13-36 months post-transplant. 1 of 3 patients with MLC compatibility and the 1 patient whose CML transformed before transplantation into ALL are alive 15 and 29 months post-transplant, respectively. Thus, 10 of the 11 patients (45%) who were excluded from the analyses are alive 14-46 months after transplantation.

Discussion

The results presented here show that optimum results of allogeneic BMT are achieved when CML patients are transplanted in first chronic phase. Similar tentative conclusions were reached in previously published reports on smaller series of patients.12,13 Although the survival
Child Health

A CLICKING HIP IN A NEWBORN BABY SHOULD NEVER BE IGNORED

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Summary

7864 infants were examined within 48 h of birth by junior medical staff. 54 with dislocation, 16 with dislocatable hip, and 25 with joint laxity were referred for abduction splinting. 622 of the infants (7.9%) had minor signs (clicking or grating hip). When these 622 infants underwent clinical and radiological examination at 4 months of age, 34 had serious hip pathology (5 dislocation, 5 dislocatable hip, and 24 radiological abnormalities). Of the 716 infants considered normal in the newborn period, 7 were referred for orthopaedic opinion (1 with dislocation and 4 with subluxation) at age 2-9 months by general practitioners and community health physicians. Thus, dislocated or dislocatable hips were 39 times more frequent in infants who had minor signs on examination within 48 h of birth than in infants considered normal. The implication of these findings is that clicking and grating of the hip are important signs which require systematic follow-up, with radiological examination at 4-6 months.

INTRODUCTION

SUCCESSFUL treatment of congenital dislocation of the hip (CDH) usually requires early diagnosis. Everyone agrees...

D. SPEER AND OTHERS: REFERRAL IN CONGENITAL DISLOCATION OF THE HIP


Survival With Bone Marrow Transplantation Versus Hydroxyurea or Interferon for Chronic Myelogenous Leukemia


Hydroxyurea, Interferon, and HLA-identical sibling bone marrow transplantation are common therapies for chronic myelogenous leukemia (CML) in chronic phase. Which is best is controversial. The purpose of this study was to compare survival of patients with CML receiving HLA-identical sibling transplant versus hydroxyurea or Interferon. The transplant cohort included 548 recipients of HLA-identical sibling transplant patients, reported to the International Bone Marrow Transplant Registry. The nontransplant cohort included 196 patients receiving hydroxyurea (n = 123) or interferon (n = 75) on a randomized trial of the German CML Study Group. Survivors were compared using proportional hazards regression with fixed and time-dependent variables to adjust for patient differences and changing risks over time. For the first 18 months after diagnosis, mortality was higher in the transplant than the nontransplant cohort (relative risk [RR], 5.85; P < .0001). From 18 to 56 months, mortality was similar (RR, 0.80; P = .38). After 56 months, mortality was lower in the transplant cohort (RR, 0.16; P < .0001). Seven-year survival probabilities (95% confidence interval) were 59% (50% to 66%) with transplant and 32% (22% to 41%) with hydroxyurea or interferon. There was a significant survival advantage for hydroxyurea or Interferon in the first 4 years after diagnosis and for transplants starting 5.6 years after diagnosis. For transplants done within 1 year of diagnosis, the survival advantage for transplantation began earlier. Survival advantage for transplants was greater and occurred earlier in patients with intermediate- and high-risk prognostic features than in those with low-risk features. This study confirms higher early mortality, but a long-term survival advantage for HLA-identical sibling transplant over hydroxyurea or interferon in CML.

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OPTIMAL THERAPY for chronic myelogenous leukemia (CML) in chronic phase is controversial. Interferon and/or hydroxyurea are common treatments. Although hydroxyurea controls white blood cell and platelet levels, it rarely produces cytogenetic remissions, prolongs survival only modestly, and does not cure.1,2 Interferon also controls white blood cell and platelet levels; however, in contrast to hydroxyurea, it produces cytogenetic remissions in some patients. In some patients, treatment with interferon survive longer than those treated with hydroxyurea or busulfan.3-10 It is too early to know if there are cures with interferon, but the frequency will be low. Treatment-related mortality with hydroxyurea and/or interferon is negligible; median survival is 3 to 7 years. Features at diagnosis associated with shorter survival include older age, male sex, high levels of myeloblasts and platelets, and splenomegaly.11-13

HLA-identical sibling bone marrow transplantation is also used to treat CML in chronic phase, especially in younger patients. Five-year leukemia-free survival is 50% to 60%.14-20 This contrasts with hydroxyurea therapy where there is no leukemia-free survival and with interferon therapy where 5-year leukemia-free survival is rare. However, transplants are associated with 20% to 30% treatment-related mortality. Factors associated with lower leukemia-free survival after chronic phase transplants include older age, prior treatment with busulfan, T-cell depletion of donor bone marrow, and intervals from diagnosis to transplant greater than 1 year.15-23

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Because most persons with CML can expect 4 or more years survival with hydroxyurea or interferon, the decision to do a transplant early, with its attendant risk of treatment-related mortality, is difficult. However, delaying transplant increases transplant-related mortality and decreases the likelihood of cure.10,11 We compared survival of 548 patients with CML receiving HLA-identical sibling transplants in chronic phase and reported to the National Bone Marrow Transplant Registry (NBMT) with survival of 196 treated with hydroxyurea or interferon on a randomized trial of the German CML Study Group.

MATERIALS AND METHODS

Transplant cohort. The study included 548 patients with CML in chronic phase, ≥15 and ≤55 years of age, diagnosed between 1983 and 1991, treated with interferon or without hydroxyurea (n = 131) or hydroxyurea alone (n = 417) followed by a non-T-cell depleted HLA-identical sibling bone marrow transplant with posttransplant methotrexate and cytosine arabinoside for graft-versus-host disease prophylaxis. Patients were reported to the NBMT by 116 centers (Table 1). Median interval between diagnosis and transplant was 10.1 (range, 2 to 84) months; 331 (65%) patients were transplanted within 1 year of diagnosis. Median follow-up was 4.3 years.

The NBMT is a voluntary working group of over 300 transplant centers worldwide that contribute data on their allogeneic and identical twin bone marrow transplants to a Statistical Center at the Medical College of Wisconsin.28 Participants are required to report all consecutive transplants. The NBMT database includes 40% to 45% of allogeneic transplant recipients since 1970. Computerized error checks, physician review of submitted data, and on-site audits of participating centers ensure data quality. Transplant outcomes estimated using NBMT data are similar to those reported by large nonparticipating centers for comparable patients.

Nontransplant cohort. The study included 196 patients with CML in chronic phase, ≥15 and ≤55 years of age, diagnosed between 1983 and 1990 and treated with hydroxyurea (n = 121) or interferon (n = 75) on a randomized trial of the German CML Study Group comparing busulfan, hydroxyurea, and interferon for newly diagnosed CML in chronic phase.22 Details of this trial are published; it enrolled about 10% of the CML cases in West Germany over a 7.5-year period. Patients were treated in 60 centers. In the trial, patients of patients treated with busulfan was inferior to those treated with either hydroxyurea or interferon; there was not a statistically significant difference in survivals of patients receiving hydroxyurea or interferon. Patients receiving busulfan were excluded from the current study. Ninety-five patients receiving hydroxyurea or interferon, but older than 55 years at diagnosis, and 14 with greater than 10% circulating blasts at diagnosis were also excluded from analysis. Median follow-up was 6.5 years. Sixty-five patients in the German study received a transplant at a median interval of 20.0 (range, 3.1 to 77.4) months after diagnosis; they were censored at time of transplant.

Statistical methods. Characteristics of the transplant and hydroxyurea/interferon groups were compared using the x2 test for categorical variables and the Wilcoxon two-sample test for continuous variables.

Comparing outcomes in the transplant and nontransplant groups required adjustment for two sources of bias: differences in time to treatment (time to transplant) and differences in baseline characteristics of patients. To address the first source of bias, which results from the fact that patients must survive in chronic phase a sufficient length of time for a transplant to be done, a left-truncated Cox regression model of time to death was used.20 At each time point in this model, the risk set in the nontransplant cohort consists of all patients still under study, while the risk set in the transplant cohort includes only those with a waiting time to transplant less than the current time point and who are still under study.

To adjust for differences in baseline characteristics, we used two approaches. First associations between survival and potential prognostic variables were evaluated in each group separately by Cox proportional hazards regression with a backwards stepwise approach. Variables considered were: age (≥ v <55 years), sex, white blood cell count (<100, 100 to 199, and ≥200 × 10/L), platelets (<150, 150 to 699, and ≥700 × 10/L), blasts (0, 1 to 3, ≥4%), spleen size (0, 1 to 4, 5 to 9, and ≥10 cm below the costal margin), hemoglobin at diagnosis (≥ v 12 g/dL) and year of diagnosis (< and ≥1988). All of these have been reported to affect survival in conventional treated patients in prior studies. Variables significantly associated with survival in either the transplant or nontransplant cohorts were included as covariates in subsequent survival comparisons. When tests for interaction between significant covariates and type of treatment (transplant vs hydroxyurea or interferon) showed significance, covariates were adjusted separately for each treatment. The proportionality assumption of the Cox model was tested by adding a time-dependent covariate for each covariate. The proportionality assumption did not hold for treatment effect, indicating that the relationship between treatment and outcome differed over time.

To determine regions of the treatment period where the relative risk (RR) of mortality between the two treatment groups was constant, a series of Cox models with different cut-off points for time-dependent treatment effects were fit.21 The final model chosen was the one giving the largest partial likelihood. In this model, treatment was considered as a time-dependent covariate with different coefficients for 0 to 18, >18 to 56, and >56 months after diagnosis. Adjusted probabilities of survival were then generated from the final Cox model stratified on treatment and weighted averages of covariate values using the sample proportion as the weight function. Adjusted probabilities represent predicted outcomes for similar groups of patients receiving each treatment. Estimates and 95% point-wise confidence intervals for differences in survival were obtained by computing estimates of survival for each treatment group separately using the sample proportion as the weight function.

The second strategy to adjust for differences in baseline characteristics was to analyze the impact of Sokal score on outcome in the transplant and nontransplant cohorts and stratify comparisons of treatment effects by Sokal risk group. Sokal score is a widely used predictor of survival in conventional treated CML.22-25 Comparisons in each stratum also used left-truncated Cox regression with time-dependent treatment effects as described above. Survival probabilities for each stratum were calculated using the left-truncated Kaplan-Meier method.

RESULTS

Patient characteristics. Table 1 compares characteristics of patients receiving transplants with those of patients receiving hydroxyurea or interferon. There were significant differences between the cohorts in distributions of age, white blood cell counts, spleen size, percent blasts in the blood, and year of diagnosis. Table 2 shows results of analyses evaluating associations between patient characteristics and survival. Age, sex, spleen size, and year of diagnosis were significant predictors. Tests of interaction indicated a differential effect of year of diagnosis in the transplant and nontransplant cohorts.
diagnosis. Using Cox regression, we identified three discrete time periods after diagnosis with relative risks of death for transplant versus nontransplant treatment that differed: ≤18, >18 to 56, and >56 months. In the first 18 months after diagnosis, patients in the transplant cohort had a higher risk of death than those receiving hydroxyurea or interferon (RR, 5.85; \( P < .0001 \)). Between 18 and 56 months, the risk of death was similar for patients in the two cohorts (RR, 0.80; \( P = .38 \)). After 56 months, patients not at risk in the transplant cohort had a lower subsequent risk of death than those still at risk in the nontransplant cohort (RR, 0.16; \( P < .0001 \)). Figure 1 shows adjusted probabilities of survival after diagnosis, calculated from the Cox models and adjusting for time to transplant, age, sex, spleen size, and year of diagnosis. The 7-year probability of survival (95% confidence interval) was 58% (50% to 65%) with transplant and 32% (22% to 41%) with hydroxyurea or interferon. Figure 2 shows differences in survival probabilities between the two cohorts (survival probability with transplant minus survival probability with hydroxyurea or interferon) over time with 95% point-wise confidence intervals for the differences. Differences greater than zero indicate a survival advantage for transplants at that point in the disease course; those less than zero indicate a survival advantage for hydroxyurea or interferon. The 95% confidence intervals that do not include zero indicate significantly different outcomes. There was a statistically significant survival advantage for hydroxyurea or interferon in the first 2.5 years after diagnosis and a significant advantage for transplants after 5.5 years; survivals were similar between 2.5 and 5.5 years.

Transplants within 1 year of diagnosis. We repeated these analyses, restricting the transplant cohort to 331 patients transplanted ≤ 1 year after diagnosis. These patients have earlier risks of transplant-related death, but better long-term transplant outcomes. Again, the RR of death in the transplant and nontransplant cohorts differed over time. In the first 18 months after diagnosis, the transplant cohort had a higher risk of death than the nontransplant cohort (RR, 5.01; \( P < .0001 \)). Between 18 and 56 months after diagnosis, the RR was 0.42 (\( P = .005 \)), and after 56 months, 0.08 (\( P = .0005 \)). Figure 3 shows adjusted probabilities of survival after diagnosis, calculated from the Cox models. The 7-year probability of survival was 67% (56% to 79%) with a transplant within 1 year of diagnosis and 30% (21% to 40%) with hydroxyurea or interferon. Figure 4 shows differences in survival probabilities between the two cohorts (transplant in the first year – hydroxyurea or interferon) over time. There was a significant survival advantage for chemotherapy in the first 1.8 years after diagnosis and a significant advantage for transplant after 4.8 years.

Association between Sokal score and outcome. Sokal scores based on sex, spleen size, hematocrit level, platelet count, and percent of blasts in the blood at diagnosis were assigned to each patient in the two cohorts (Table 1). There were insufficient data to assign a Sokal score to 211 transplant recipients; however, survival of these patients was similar to the 337 transplant recipients assigned a Sokal score (Fig 5A). Patients were classified as low-, intermediate-, and high-risk as published. Survival in the transplant cohort did not differ among the three risk groups (Fig 5A). Transplant patients were not, therefore, stratified by risk group in subsequent comparisons. In the nontransplant cohort, low-risk patients had significantly longer survivals than intermediate- and high-risk patients, who were similar to each other (Fig 5B).

Figure 6A shows probabilities of survival after diagnosis for transplant recipients and low-risk patients receiving hydroxyurea or interferon. The 7-year probability of survival was 58% (52% to 64%) with transplant and 49% (34% to 63%) with hydroxyurea or interferon. Although the curves cross at about 6 years, there was not a statistically significant advantage for transplant until 7.8 years after diagnosis. Figure 6B compares probabilities of survival for patients receiving transplant within 1 year of diagnosis with low-risk patients receiving hydroxyurea or interferon. The 7-year probability of survival was 67% (60% to 73%) with transplant and 49% (34% to 63%) with hydroxyurea or interferon. The curves cross at 5 years, with a

Table 1. Comparison of Transplant and Nontransplant Cohorts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Transplant, No.</th>
<th>Hydroxyurea or Interferon, No.</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>548</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Age, median (range)</td>
<td>548</td>
<td>35 (15-54)</td>
<td>196 41 (15-55)</td>
</tr>
<tr>
<td>Male sex, No. (%)</td>
<td>548</td>
<td>331 (60)</td>
<td>196 119 (61)</td>
</tr>
<tr>
<td>WBC at Dx, median</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(range)</td>
<td>548</td>
<td>140 (3-890)</td>
<td>196 150 (15-489)</td>
</tr>
<tr>
<td>Hemoglobin at Dx, median (range)</td>
<td>479*</td>
<td>12 (2-17)</td>
<td>195 12 (4-16)</td>
</tr>
<tr>
<td>Platelets at Dx, median (range)</td>
<td>466*</td>
<td>3 (0-26)</td>
<td>196 5 (0-30)</td>
</tr>
<tr>
<td>N x 10^9/pl</td>
<td>461*</td>
<td>3 (2-3,440)</td>
<td>196 412 (54-1,735)</td>
</tr>
<tr>
<td>Spleen size at Dx, cm</td>
<td>123 (47)</td>
<td>72 (27)</td>
<td></td>
</tr>
<tr>
<td>Intermediate risk</td>
<td>125 (37)</td>
<td>83 (42)</td>
<td></td>
</tr>
<tr>
<td>High risk</td>
<td>75 (22)</td>
<td>41 (21)</td>
<td></td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td>1988, No. (%)</td>
<td>548 294 (44)</td>
<td>106 94 (48)</td>
</tr>
</tbody>
</table>

*Not available for all patients.
Abbreviations: WBC, white blood cell count; Dx, diagnosis.

Table 2. Variables Associated With Survival in the Transplant and/or Nontransplant Cohorts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Transplant, RR of Death</th>
<th>Nontransplant, RR of Death</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year diagnosis ≥88</td>
<td>0.58</td>
<td>0.003</td>
<td>NS</td>
</tr>
<tr>
<td>Spleen size ≥10 cm</td>
<td>0.85</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Female sex</td>
<td>0.75</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td>Age &gt;35 years</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other variables considered: platelets at diagnosis (0 to 149, 150 to 659, ≥700 x 10^9/μL, unknown), circulating blasts at diagnosis (0, 1 to 3, ≥4, unknown), WBC at diagnosis (<100, 100 to 199, ≥200 x 10^9/μL), Abbreviations: RR, relative risk; NS, not significant.
Fig 1. Adjusted probabilities (from Cox regression model) of survival after diagnosis of CML in persons receiving HLA-identical sibling bone marrow transplants or nontransplant therapy with hydroxyurea or interferon.

Statistically significant advantage for transplant after about 6.5 years from diagnosis.

Figure 7a shows probabilities of survival after diagnosis for transplant recipients and intermediate- and high-risk patients receiving hydroxyurea or interferon. The 7-year probability of survival was 58% (52% to 64%) with transplant and 21% (12% to 31%) with hydroxyurea or interferon. The curves cross at 3.5 years, with a statistically significant advantage for transplant after 4.7 years. Figure 7b compares probabilities of survival for patients receiving transplant within 1 year of diagnosis with intermediate- and high-risk patients receiving hydroxyurea or interferon. The 7-year probability of survival was 67% (63% to 73%) with transplant and 21% (12% to 31%) with hydroxyurea or interferon. The curves cross at 2.2 years, with a statistically

Fig 2. Differences (with 95% confidence interval) in adjusted probabilities of survival after diagnosis of CML between patients receiving HLA-identical sibling bone marrow transplants versus hydroxyurea or interferon. Differences were calculated as probability of survival with transplant minus probability of survival with nontransplant treatment. A negative difference indicates a survival disadvantage for transplant; a positive difference indicates an advantage for transplants. A 95% confidence interval that does not include zero indicates a statistically significant difference.
significant advantage for transplants after about 4 years from diagnosis.

DISCUSSION

The best treatment for CML in chronic phase is controversial. HLA-identical sibling bone marrow transplants can cure a substantial proportion of patients with CML, but have high treatment-related mortality. In contrast, hydroxyurea and interferon have little treatment-related mortality and prolong survival, but cure few, if any, patients. This study confirms the initial survival disadvantage of transplants, but also the later advantage; the latter becomes significant between 4 and 8 years after diagnosis, depending on when in chronic phase transplants are performed and disease-related prognostic factors. Importantly, the early survival disadvantage of transplants is increased and the later survival advantage is decreased by delaying
transplants beyond the first year after diagnosis. This results from increased treatment-related mortality and relapse with transplants done later.16,21

The nontransplant cohort in this study included patients receiving hydroxyurea or interferon, treatments with comparable survival in the data set we used.5 This study set differs from other reports in which interferon treatment seemed to have better survival than hydroxyurea in nonrandomized and randomized settings. Differences between the German CML Study and these other studies were recently discussed in detail, including on-study criteria, risk profiles, drug doses, and schedules and frequency of cytogenetic testing.13,26 However, the 62-month median survival of the hydroxyurea and interferon cohort in this study is similar to that reported in other studies of interferon (55 to 72 months) so the comparison between transplant and nontransplant therapy applies to most patients with CML who are <55 years old. Additionally, the late advantage of transplants is seen even when compared with nontransplant therapy.
in low-risk patients in the German trial, who may be more similar to those in other interferon trials. Whether a strategy of early transplant for interferon responders and delayed transplant for interferon responders would improve survival can only be determined in a prospective trial.

The only type of transplant we studied were those from HLA-identical siblings. Because treatment-related mortality after transplants from other related and unrelated donors is substantially higher, our conclusions may not apply to these settings. It is likely that the time point after which alternative donor transplants show better survival over hydroxyurea or interferon is later.

This study confirms a long-term survival advantage for transplants and further indicates that the survival advantage is greatest for persons with intermediate- and high-risk prognostic features at diagnosis and when transplants are performed early. It quantifies the trade-off between early mortality and long-term leukemia-free survival with transplants versus hydroxyurea or interferon for newly diagnosed CML and provides data for counseling patients deciding between therapies.
Fig 7. Adjusted probabilities of survival after diagnosis of CML in intermediate- and high-risk persons receiving hydroxyurea or interferon versus persons receiving an HLA-identical sibling bone marrow transplant (A) at any time after diagnosis or (B) within 1 year of diagnosis.

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Bone Marrow Transplantation for Chronic Myelogenous Leukemia in Chronic Phase

Increased Risk for Relapse Associated with T-Cell Depletion

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Data on 405 patients with chronic myelogenous leukemia who received bone marrow transplants in chronic phase were analyzed for factors predictive of outcome. The 4-year actuarial probability of relapse was 13% (95% confidence interval [CI], 12% to 28%) and of survival, 55% (95% CI, 50% to 60%). In multivariate analyses the probability of relapse was higher for recipients of T-cell-depleted bone marrow compared with recipients of non-T-cell-depleted bone marrow (relative risk, 5.4; \( P < 0.0001 \)) and for patients who did not develop chronic graft-versus-host disease with patients who did (relative risk, 3.1; \( P < 0.01 \)). The probability of survival was lower for patients who developed moderate to severe acute graft-versus-host disease than for patients with no or mild acute graft-versus-host disease (relative risk, 2.6; \( P < 0.0002 \)). Duration of disease before transplant was not associated with outcome. Bone marrow transplantation done in the chronic phase of chronic myelogenous leukemia offers some patients prolonged leukemia-free survival. The T-cell-depleted grafts are associated with an increased probability of relapse.

Patients with chronic myelogenous leukemia in chronic phase usually receive treatment to improve their clinical condition, but there is little evidence that it prolongs the chronic phase or that overall survival is increased. Until recently patients with chronic myelogenous leukemia could not be cured. Even when the leukocyte count is reduced to normal, cytogenetic abnormalities such as the Philadelphia chromosome indicate persistent leukemia. The chronic phase evolves into the accelerated or acute (blast) phase in a median of 3 years.

The advanced phases usually are fatal within 3 to 12 months.

Experience with high-dose chemoradiotherapy and bone marrow transplantation, initially with genetically identical twin donors (1) and more recently HLA-identical sibling donors (2-10), shows that long-term leukemia-free survival can be achieved in many patients. Disease status at transplantation is strongly correlated with outcome. Recipients of transplants in chronic phase have a higher probability of survival and lower risk of relapse than recipients of transplants with advanced disease (7-10).

Data from 405 patients with chronic myelogenous leukemia in chronic phase were analyzed to identify factors predicting relapse and survival. All patients received bone marrow transplants from HLA-identical siblings. We address two issues: when transplants should be done in chronic phase and the effect of T-cell depletion on outcome.

The risk for transformation and death in patients with chronic myelogenous leukemia who receive conventional treatment is approximately 10% in each of the first 2 years after diagnosis and 20% to 25% per year thereafter (11). In contrast, transplantation, although currently the only possibility of cure, is associated with substantial early morbidity and mortality. These facts make it difficult for the physician to decide when in chronic phase to recommend transplantation. A recent report (9) suggests that results are best when transplantation is done early in chronic phase. We tested this finding in a large, independent patient population.

One major reason for mortality associated with allogeneic bone marrow transplantation is acute graft-versus-host disease. To reduce the risk for this complication, several centers have depleted donor bone marrow of T cells before infusion. However, this depletion may decrease the postulated antileukemic effect of allogeneic bone marrow transplantation (12, 13). Thus, an increased risk for leukemia relapse might offset the benefit of a decreased risk for graft-versus-host disease. We therefore also evaluated the effect of T-cell depletion on outcome.

01988 American College of Physicians
Patients and Methods

PATIENTS

Data for 455 patients with chronic myelogenous leukemia in first chronic phase, receiving bone marrow transplants between 1 July 1978 and 31 December 1985, were reported to the International Bone Marrow Transplant Registry by 82 transplant teams in 27 countries. Excluded were 36 recipients of bone marrow from donors other than HLA-identical siblings, 12 recipients of bone marrow from genetically identical twins, and 2 patients with juvenile chronic myelogenous leukemia. This study was restricted to 405 recipients of bone marrow from HLA-identical siblings. The closing date was 30 September 1986. The median follow-up time was 25 months (range, 4 months to 6.4 years).

Most patients (98%) were prepared for transplantation with high-dose chemotherapy and total body radiation administered as a single dose (38%) or in several fractions (62%). Seven patients received chemotherapy alone and one patient received radiation therapy alone. Eighty-seven patients from 20 centers received donor bone marrow cells that had been depleted of T-cells in vitro to prevent or modify graft-versus-host disease. The depletion involved incubation of donor marrow with one or more monoclonal antibodies with or without complement in 76 patients. Other methods, such as sheep erythrocyte rosetting with or without lectins and elutriation, were used in 11 patients.

Sixty-three of eighty-seven (72%) recipients of T-cell-depleted grafts also received cyclosporine after transplant to prevent graft-versus-host disease; 2 (2%) received methotrexate; and 22 (25%) received no additional prophylaxis. Of the 318 patients receiving non-T-cell-depleted bone marrow 96 (30%) received methotrexate; 200 (63%), cyclosporine; 16 (5%), both methotrexate and cyclosporine; 1 (0.3%) corticosteroids alone; and 5 (1%) no prophylaxis against graft-versus-host disease. Acute graft-versus-host disease was classified as absent, mild, moderate, moderately severe, or severe using previously published criteria (14). The reproducibility of this approach among centers has been extensively studied (15). Signs and symptoms associated with chronic graft-versus-host disease were classified as absent, mild, moderate, or severe. An overall score for chronic graft-versus-host disease was generated by the transplant team and verified at the Statistical Center of the Registry.

Relapse of leukemia was defined by hematologic criteria and supported by cytogenetic findings, that is, recurrence of the Philadelphia chromosome. However, reappearance of the Philadelphia chromosome alone was not scored as relapse because the biologic importance of such cytogenetic changes is not yet established (9, 10, 16, 17). Furthermore, the frequency with which recurrence of the Philadelphia chromosome is recognized varies because it depends on the frequency of cytogenetic examinations of bone marrow and number of metaphases examined.

Statistical Methods

Thirty variables (Table 1) were studied in univariate analyses for possible association with relapse or survival. Chi-squared analysis was used to test differences between groups in discrete variables and Student t-test was used to test differences in continuous variables. Actuarial curves for relapse and survival were prepared using standard life tables. Curves were truncated when fewer than three patients remained at risk. Curves showing outcome according to whether patients developed acute graft-versus-host disease were derived from life-table analyses of patients who survived at least 1 day after transplant with evidence of engraftment, and were at risk for acute graft-versus-host disease. Curves showing the probability of relapse according to whether patients developed chronic graft-versus-host disease were derived from life-table analyses of patients who survived at least 21 days after transplant with evidence of engraftment, and were at risk for chronic graft-versus-host disease. Only 6 of 123 (5%) patients who developed chronic graft-versus-host disease did so before this time.

Because most transplants using T-cell-depleted marrow were done after 1982, data at 2 or 3 rather than 4 years were used to compute probability of relapse among recipients of such grafts. Variables associated with relapse or survival in univariate analyses with P < 0.10 were studied in multivariate analyses using Cox proportional hazards regression models (18). Acute and chronic graft-versus-host disease were entered into the regression equation as time-dependent covariates; that is, patients were scored as having acute or chronic graft-versus-host disease only after the time of its occurrence. Relative risks for relapse or death associated with particular prognostic factors were derived from the proportional hazards models.

All multivariate analyses were examined and adjusted for possible center effects both by treating transplant center as a covariate in the regression model, and by using a proportional hazards model stratified by center (19). The relative risks and statistical significance of prognostic variables were virtually identical with and without these adjustments. All P values are two-tailed and, unless specified, based on the results of multivariate analyses. Because comparisons of several factors were made, only P < 0.01 was considered significant; P values between 0.01 and 0.05 were considered marginally significant and are presented only to show trends.

Results

Probability of Relapse and Survival

The 4-year probability of relapse in 405 patients was 19% (95% CI, 12% to 28%), the 4-year probability of survival was 55% (95% CI, 50% to 60%), and the 4-year probability of leukemia-free survival was 46% (95% CI, 40% to 52%). Two hundred and nine patients (52%) are in continuous complete remission, and 27 (7%) are in chronic phase after relapse of chronic myelogenous leukemia. One hundred and sixty-nine patients have died, only 5 (1%) of leukemia. Causes of death are shown in Figure 1.

Factors Associated with Relapse

Six variables were associated with the probability of relapse in univariate analyses with P < 0.10 (Table 1). Two factors remained significant in multivariate analyses: the method of graft-versus-host disease prophylaxis and the presence or absence of chronic graft-versus-host disease.

The 3-year probability of relapse for 318 recipients of non-T-cell-depleted bone marrow was 9% (95% CI, 5% to 15%) compared with a probability of 48% (95% CI, 32% to 65%) for 87 recipients of T-cell-depleted marrow (relative risk, 5.4; P < 0.0001). There was no indication of biased selection of patients to receive or not receive T-cell-depleted grafts. At 15 centers, T-cell-depleted grafts were done sequentially, and at 5 centers according to predetermined protocols. Patient and treatment characteristics as well as outcomes after transplant of recipients of T-cell-depleted and non-T-cell-depleted bone marrow are shown in Table 2. Recipients of T-cell-depleted marrow were significantly older than recipients of non-T-cell-depleted marrow, and more likely to receive splenic radiation, fractional rather than single-dose radiation, and radiation at higher dose rates. Patients were also less likely to have had splenectomy, intrathecal chemotherapy, or trimethoprime-sulfamethoxazole before transplantation. Adjustment of multivariate analysis for these differences did not significantly alter the relative risk of relapse associated with T-cell depletion.

Goldman et al. • Bone Marrow Transplantation
<table>
<thead>
<tr>
<th>Variables</th>
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* Associated with the probability of relapse at \( P < 0.01 \) level.
† Associated with the probability of relapse at \( P < 0.001 \) level.
‡ Among patients surviving 31 or more days after transplant with evidence of engraftment.
§ Among patients surviving 31 or more days after transplant with evidence of engraftment.

In univariate analysis, T-cell depletion was associated with a decreased incidence of moderate to severe acute graft-versus-host disease (15% in recipients of T-cell-depleted marrow compared with 51% in recipients of non-T-cell-depleted marrow; \( P < 0.0001 \)), of chronic graft-versus-host disease (19% compared with 39%, respectively; \( P < 0.003 \)), and of death from graft-versus-host disease (7% compared with 27%, respectively; \( P < 0.0002 \)). To determine whether the increased risk of relapse associated with T-cell depletion was mediated through its effect on graft-versus-host disease, patients with and without acute and chronic graft-versus-host disease were analyzed separately (Figure 2). An increased risk of relapse was seen in recipients of T-cell-depleted marrow regardless of whether clinically evident acute or chronic graft-versus-host disease developed.

There was no significant difference (\( P = 0.34 \)) in the 2-year probability of relapse among 22 patients who received T-cell-depleted marrow alone (30% [95% CI, 11% to 59%]) compared with the 63 patients who received T-cell-depleted marrow and cyclosporine (48% [95% CI, 29% to 67%]). Duration of follow-up in patients who received T-cell-depleted marrow without cyclosporine was inadequate to allow calculation of relapse probability beyond 2 years. Among recipients of non-T-cell-depleted bone marrow, the probability of

---

Figure 1. Principal causes of death after transplantation in patients with chronic myelogenous leukemia in chronic phase. If leukemia was present at death, it is shown as the cause regardless of underlying or concomitant cause. IPN = interstitial pneumonitis; GVHD = graft-versus-host disease. "Other" includes death from organ failure (7), veno-occlusive disease (7), bone marrow aspiration (3), pulmonary embolism (2), acute encephalopathy (1), suicide (1), intestinal volvulus (1), and drug toxicity (1).
Table 2. Comparison of Characteristics of Recipients of T-cell-Depleted and Non-T-cell-Depleted Bone Marrow

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Distribution or Frequency</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>33 (9-50)</td>
<td>29 (3-52)</td>
</tr>
<tr>
<td>Median leukocyte count at diagnosis, x 10⁹/L (range)</td>
<td>138 (5.6-620)</td>
<td>150 (15.4-682)</td>
</tr>
<tr>
<td>Median performance score pretransplant, % (range)</td>
<td>100 (70-100)</td>
<td>100 (70-100)</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>Philadelphia chromosome present, %</td>
<td>93</td>
<td>95</td>
</tr>
<tr>
<td>Median interval from diagnosis to transplant, mos (range)</td>
<td>15 (4-117)</td>
<td>17 (1-144)</td>
</tr>
<tr>
<td>Sero-mismatch, %</td>
<td>Male to male</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Male to female</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Female to female</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Female to male</td>
<td>26</td>
</tr>
<tr>
<td>Median dose of irradiation, Gy (range)</td>
<td>10.2 (5.5-15.75)</td>
<td>10.0 (5.5-15.3)</td>
</tr>
<tr>
<td>Median dose-rate, Gy/min (range)</td>
<td>12.5 (2.4-26)</td>
<td>6.8 (1.9-52)</td>
</tr>
<tr>
<td>Radiation fractionated, %</td>
<td>76</td>
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</tr>
<tr>
<td>Intrathecal chemotherapy, %</td>
<td>6</td>
<td>26</td>
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<tr>
<td>Splenectomy before transplant, %</td>
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<td>13</td>
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<tr>
<td>Splenic radiation before transplant, %</td>
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<td>13</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole prophylaxis, %</td>
<td>52</td>
<td>74</td>
</tr>
<tr>
<td>Graft failure, %</td>
<td>7</td>
<td>1</td>
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<tr>
<td>Moderate to severe acute graft-versus-host disease, %</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>Case-fatality, %</td>
<td>25</td>
<td>39</td>
</tr>
<tr>
<td>Mild to severe chronic graft-versus-host disease, %</td>
<td>20</td>
<td>38</td>
</tr>
<tr>
<td>Intestinal pneumonitis, %</td>
<td>23</td>
<td>27</td>
</tr>
<tr>
<td>Case-fatality, %</td>
<td>75</td>
<td>79</td>
</tr>
</tbody>
</table>

* All analyses are seven variance contrasted variables compared using Student's t-test; compared variables compared using chi-squared test. NS = not significant.

Relapse was not significantly different between patients receiving methotrexate (7% [95% CI, 3% to 17%]) or cyclosporine (10% [95% CI, 6% to 17%]) to prevent graft-versus-host disease.

The absence of chronic graft-versus-host disease was associated with a higher probability of relapse at 4 years (24% [95% CI, 16% to 34%]) compared to patients surviving more than 51 days (after transplant) than the occurrence of chronic graft-versus-host disease (6% [95% CI, 3% to 13%]; relative risk, 3.1; P < 0.01). Development of chronic graft-versus-host disease was associated with a decreased risk of relapse whether or not the patient had received T-cell-depleted bone marrow (Figure 2, right).

A higher 4-year probability of relapse was seen in 340 patients 20 years of age or older (23% [95% CI, 14% to 35%]) compared to younger patients (8% [95% CI, 3% to 21%]), but this finding had only borderline significance (P < 0.03).

We studied whether the interval from diagnosis to transplant influenced the likelihood of relapse. At 4 years after transplant, the probability of relapse was 17% (95% CI, 9% to 29%) in 136 patients receiving a transplanted bone marrow.

---

**Figure 2.** Life-table analyses of actuarial probability of relapse of patients with chronic myelogenous leukemia in chronic phase remaining in remission. The number of patients living and available for analysis at each time point is shown in parentheses. Figure 2, left. Figure shows whether bone marrow was or was not replaced with T cells and whether acute graft-versus-host disease (AGVHD) developed in patients at risk. Figure 2, right. Figure shows whether donor bone marrow was or was not replaced with T cells and whether chronic graft-versus-host disease (C GVHD) developed in patients at risk.
plant within 1 year of diagnosis. 28% (95% CI, 15% to 46%) in 181 patients receiving a transplant 1 to 3 years after diagnosis, and 13% (95% CI, 6% to 26%) in 87 patients receiving a transplant more than 3 years after diagnosis. These differences were not statistically significant.

**FACTORS ASSOCIATED WITH SURVIVAL**

Seven variables associated with survival in univariate analyses with $P < 0.10$ (Table 1) were included in multivariate analyses. Two variables remained significant: severity of acute graft-versus-host disease and age of the patient (Figure 3, left).

Moderate to severe acute graft-versus-host disease was strongly associated with mortality. Of the 395 patients surviving at least 21 days with engraftment, and at risk for acute graft-versus-host disease, the 4-year probability of survival was 74% (95% CI, 68% to 79%) for the 221 patients with no or mild acute graft-versus-host disease and 35% (95% CI, 28% to 43%) for the 174 patients with moderate to severe acute graft-versus-host disease (relative risk, 3.7; $P < 0.0001$).

Patient age was another determinant of survival and was a significant risk factor whether analyzed as a continuous variable, by decade (Table 1), or as a dichotomous variable (Figure 3, left). The 4-year probability of survival for 65 patients less than 20 years of age was 75% (95% CI, 63% to 84%); this was significantly higher than the 52% (95% CI, 46% to 58%) probability for 340 older patients (relative risk, 2.6; $P < 0.0002$). Older patients had higher mortality rates due to acute graft-versus-host disease and interstitial pneumonitis than younger patients. The incidence of interstitial pneumonitis was similar in older and younger patients (27% compared with 22%, respectively); however, the case-fatality rate was significantly higher in older patients (82% compared with 43%; univariate $P < 0.002$). Similarly, the incidence of moderate to severe acute graft-versus-host disease was not significantly different in patients aged 20 years or older or patients less than 20 years (42% compared with 46%, respectively) but the case-fatality rate was higher among older patients (51% compared with 27%; univariate analysis $P < 0.009$). A higher probability of survival also occurred with younger donors; however, donor and recipient ages were highly correlated ($r = 0.79$) and it was impossible to determine whether donor age, recipient age, or both influenced survival.

No other variable studied, including T-cell depletion, was significantly associated with the probability of survival. There was no significant influence of duration of the disease before transplant on survival (Figure 3, right).

**LEUKEMIA-FREE SURVIVAL**

The effects of older patient age, acute and chronic graft-versus-host disease, and T-cell depletion on leukemia-free survival were studied using multivariate analysis. Patients with moderate to severe acute graft-versus-host disease had a risk for treatment failure (death or relapse) 3.2 times higher than patients with no or mild acute graft-versus-host disease ($P < 0.0001$). The probability of leukemia-free survival was decreased in patients aged 30 or older (relative risk, 2.5; $P < 0.0001$). After adjusting for the effects of age and acute graft-versus-host disease, patients with chronic graft-versus-host disease had a slightly lower risk of treatment failure (relative risk, 0.8) but this value was not statistically significant ($P = 0.23$). The T-cell depletion was not significantly
associated with leukemia-free survival in univariate or multivariate analysis (relative risk, 1.3; P = 0.19). Decreased early mortality due to reduced incidence and severity of graft-versus-host disease in recipients of T-cell-depleted marrow was offset by an increased frequency of graft failure and relapse (Table 2).

Discussion

Two factors associated with the risk of relapse are whether or not T cells were depleted from donor bone marrow as prophylaxis against graft-versus-host disease and whether or not chronic graft-versus-host disease occurred. Recipients of T-cell-depleted bone marrow had a higher probability of relapse than recipients of non-T-cell-depleted bone marrow. This finding is consistent with data in animals and humans showing an antileukemic effect associated with allogeneic bone marrow transplantation (12, 13, 20-22). This effect is believed to be due to a graft-versus-leukemia reaction mediated by transplanted T cells.

Data in humans supporting this concept include a high risk for relapse in identical twin transplants for acute myelogenous leukemia in first remission (12, 13), an inverse correlation between acute and chronic graft-versus-host disease and relapse in acute myelogenous leukemia in first remission and in advanced acute leukemias (23-25), and several reports (26-28) suggesting an increased likelihood of relapse in recipients of T-cell-depleted bone marrow compared with recipients of non-T-cell-depleted marrow. These last reports are confirmed in this analysis. It also is possible that T-cell depletion, often associated with delayed hematopoietic engraftment (29), might provide a nonimmunologic survival advantage to damaged leukemic stem cells after chemotherapy and radiation.

A decreased risk for recurrent leukemia associated with chronic graft-versus-host disease has been reported in acute leukemias (12, 13, 26). Why a significant antileukemic effect was seen with chronic but not acute graft-versus-host disease in chronic myelogenous leukemia is not known. It is possible that different subsets of T cells or mechanisms are responsible for acute and chronic graft-versus-host disease. Also, the cells responsible for the graft-versus-leukemia effect are not necessarily identical to cells responsible for graft-versus-host disease (12). Both direct (30) and indirect (31, 32) evidence derived from animal studies suggest that at least some cells responsible for graft-versus-leukemia reactions are distinct from cells causing graft-versus-host disease.

Two factors were significantly associated with survival after transplantation in chronic phase: the severity of acute graft-versus-host disease and age. These factors also adversely affected leukemia-free survival. Patients who developed no or only mild acute graft-versus-host disease had a greater chance of survival than patients with moderate to severe acute graft-versus-host disease. Younger patients had a higher probability of survival than older patients. It is important to emphasize that because both variables are significant in multivariate analysis, the relative risk for treatment failure associated with patients 20 years or older represents the effect of age over and above its association with acute graft-versus-host disease. Younger patients had significantly less mortality from acute graft-versus-host disease and interstitial pneumonitis than older patients in the first few months after transplantation. Although incidences of interstitial pneumonitis and acute graft-versus-host disease were not higher in older patients, case-fatality rates were significantly increased. The reason older patients were less likely to survive interstitial pneumonitis and acute graft-versus-host disease than younger patients is not known but may relate to relative ability to sustain and repair tissue damage.

Despite its impact on the probability of relapse, use of T-cell-depleted marrow did not significantly affect overall probability of survival or leukemia-free survival. In univariate analyses, use of T-cell-depleted marrow decreased the incidence of acute and chronic graft-versus-host disease and, therefore, death from graft-versus-host disease (Table 2). It did not alter the case-fatality rate of moderate to severe acute graft-versus-host disease, or the incidence or case-fatality rate of interstitial pneumonitis (Table 2). Use of T-cell-depleted marrow substantially increased the incidence of graft failure and death from aplasia (Table 2). These opposing effects on outcome offset each other when looking at the overall effect of T-cell depletion on survival and leukemia-free survival. Although the median follow-up of patients who received T-cell-depleted bone marrow was shorter than that for patients who received other methods of graft-versus-host disease prophylaxis, the relapse curves in Figure 2 do not show a plateau that might signify a decrease in the risk for relapse with time in these patients. If the rate of relapse does not decline with continued follow-up, the ultimate effect of T-cell depletion on long-term leukemia-free survival may be negative.

Duration of disease had no influence on relapse or survival, contrary to a previous study (9). In a series of patients who received transplants in the chronic phase of chronic myelogenous leukemia, Thomas and coworkers (9) found that 16 patients who received transplants more than 3 years after diagnosis had a significantly lower probability of survival than 24 patients who received transplants within 1 year of diagnosis. The disparity between these reports is not known. Differences in chemotherapy used before transplantation may play a role but insufficient data regarding type and amount of previous chemotherapy were available for analysis, and such differences did not appear to play a role. It is possible that we failed to detect a difference due to chance alone; however, with a sample size of 405 patients, the probability of missing a real difference of the magnitude reported in the study of Thomas and coworkers (9) is less than 1 in 100.

Two studies (10, 33) also failed to find a relation between disease duration and survival. Furthermore, most patients with chronic myelogenous leukemia referred for transplantation have not been treated by any standard protocol before transplantation. Therefore, it is unlikely that specific patterns of patient referral or the multi-institutional nature of our study account for the absence of a relation between disease duration and survival.
It is important to consider which patients with chronic myelogenous leukemia are candidates for transplantation. Because of the generally accepted adverse influence of increasing age in allogeneic bone marrow transplantation, an upper age limit might seem reasonable. Our data show that the adverse effect of older age is fully manifested by age 20 and does not seem to increase thereafter. No appreciable difference in the probability of survival was seen among patients who received transplants in the third, fourth, and fifth decades of life (Table 1).

Deciding when to recommend bone marrow transplantation for the patient with chronic myelogenous leukemia who has an HLA-identical sibling is difficult. Clearly transplantation is most successful when it is done before the onset of blast crisis, but this step cannot be predicted with certainty once transplantation has occurred. The results of bone marrow transplantation are much poorer. Because transplantation offers the only possibility of cure, the opportunity should not be lost by prolonged delay, especially in younger patients with an HLA-identical sibling.

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References


CHAPTER 4
CYCLOSPORIN-A
THE FIRST LOCAL INNOVATION
Bone marrow transplantation using HLA - identical and MLC non-reactive siblings has become the accepted form of treatment for patients with severe acute aplastic anaemia and immunodeficiency disease. This procedure also gave encouraging early results in leukaemia when transplanted in first complete remission. In a study of 30 patients with acquired bone marrow failure who had been minimally exposed to antigens contained in blood the projected survival at six years was 75%. In selected cases the incidence of graft rejection, previously in the range of 25 to 60%, was reduced. Nevertheless graft-versus-host disease still affects over half of those causing long-term problems in 20% of the survivors.

Attempts to immunologically influence this complication were undertaken by testing a unique undecapeptide molecule derived from mushroom cultures by Jean Borel working at Sandoz in Switzerland. Using the now well standardised rabbit model and establishing a radioimmunoassay benefit was shown that led to confirmatory studies in this particular context. Additional encouragement was derived from use in autoimmune diseases in acute aplasia and on preformed lymphocyte toxic antibodies in cardiac transplantation and nephrology. Turning to the clinical use of this as a single agent in severe acute aplastic anaemia it became clear that, on its own or even in combination with antilymphocyte serum, data were insufficient to recommend use as a single agent. In further studies on haematopoietic stem cell allografting it became clear that, in contrast to very substantial success in solid organ replacement benefit was present but only partial. Thus graft-versus-host disease was reduced in frequency and intensity but not completely abrogated despite pre-treatment of both donor and recipient and when tested in donor pre-treatment alone.

In studies that have continued over the last decade, scattered among centres across the globe, there has been no consensus. Rather documentation accumulated of varying effect for the use of this extraordinary non-cytotoxic immunosuppressive agent but in a somewhat random array of reports. Thus, although still currently employed in our own programs, the unusually low incidence of this unique immunologic disease following on the introduction of the Campath series of monoclonal antibodies, has led to limiting the use of cyclosporin A to the single situation of matched-unrelated volunteer donors and then only for a limited period.
CYCLOSPORIN A RADIOIMMUNOASSAY. A COMPARISON OF SERUM, PLASMA AND WHOLE BLOOD LEVELS
Gail Randall and Peter Jacobs
The University of Cape Town Leukaemia Centre and the Department of Haematology
Groote Schuur Hospital, Observatory, Cape, South Africa

Cyclosporin A is being increasingly employed as an immunosuppressive agent with indications ranging from autoimmune disease to organ transplantation. A number of unresolved issues surround the use of this new agent including the definition of immunosuppressive level in vivo and optimal methods for monitoring adequate drug dosage. In this latter context, the relative merits of high performance liquid chromatography as opposed to radioimmunoassay to attract attention while a number of variables are known to influence the use radioimmunoassay for measuring both the parent substance and its metabolites. The objective of this study was to examine the effect of temperature on separation procedures and to refine the method used for whole blood radioimmunoassay. Separation at 37°C gave plasma or serum concentrations on average 30% higher than when the centrifugation took place at ambient (22°C) temperature. In addition, the distribution of cyclosporin A between whole blood and plasma or serum was significantly different with the former being 2.5 times higher than the latter even when separation took place at 37°C. The recommended method using whole blood was found unsatisfactory because anticoagulated samples frozen and then thawed were difficult to pipette. The method was accordingly modified in one of two ways. The sample was stored at 4°C and then well mixed before use or alternatively the fresh sample was prediluted with buffer and these aliquots then frozen or held at 4°C until required. The measurement using the latter modification gave reproducible values which were not significantly different from those obtained from fresh (4°C) whole blood. However, once whole blood had been frozen and then thawed the values were lower by a factor of 0.83 when compared to either modification. It is concluded that plasma or serum samples are suitable for monitoring cyclosporin A levels but require careful separation at 37°C. Since this is not standard practice the alternative use of whole blood is preferable and in this regard samples should not be frozen and thawed but held at 4°C or alternatively prediluted with buffer after which the disadvantage of freezing no longer applies.

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PERSPECTIVES IN IMMUNOSUPPRESSION

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Professor P. van der .... Department of ..........
Cyclosporin-A Radioimmunoassay: a Modified Method for Whole Blood Determination

Gail Randall and Peter Jacobs
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(Received 1 October 1984; in revised form 11 April 1985; accepted 15 April 1985)

Abstract. Cyclosporin-A levels were determined by radioimmunoassay in plasma or whole blood, using split samples collected from patients receiving this agent as the only form of immunosuppression following allogeneic bone marrow transplantation. In the plasma assay the temperature at which centrifugation took place was critical since the mean levels were approximately 30% higher with separation at 37°C in comparison to 20°C or lower. Furthermore, the level in whole blood samples was 2.4 times higher than that from the matching serum. In addition, anticoagulated blood that had been frozen and then thawed was technically more difficult to pipette and resulted in a recovery of only 83% of the cyclosporin when compared with assay using fresh blood. In contrast, consistent measurements were obtained either when whole blood was stored at 4°C and then well mixed and diluted in buffer immediately prior to use or when such buffered samples were frozen and thawed immediately before analysis. The latter modifications render the whole blood assay a practical and reliable means for monitoring cyclosporin-A concentrations and may avoid excessive and the potentially nephrotoxic levels achieved when plasma levels are held in ranges previously considered therapeutic.

Key words: Whole blood versus serum — Radioimmunoassay — Cyclosporin A

Cyclosporin A (OL 27-400) is a cyclic decapeptide of fungal origin. The purified substance has been demonstrated in vitro to interfere with an early stage of the immune mechanism for antigen recognition, probably at the level of T-lymphocyte helper function. Predictably, such an action is potentially immunosuppressive and studies in experimental animals [1] and man [2] have confirmed this activity in organ transplantation [3], bone marrow grafting [4], and a number of immunologically mediated diseases [5]. The major toxicity of cyclosporin A is on renal function [6], although it has been incriminated in producing liver dysfunction [7].

Since the optimal immunosuppressive level remains debatable, difficulties exist on how best to balance the therapeutic dose against the development of undesirable side effects. Furthermore, cyclosporin A distributes between cellular receptors and lipoprotein in the plasma [8], and elevations in the latter level may therefore reflect drug present over and above that required to saturate tissue-binding sites. Understandably, a number of different approaches to its use have been reported. Initially, plasma or serum levels were used [9], although the recommended ranges varied widely. Since it is difficult to correlate accurately the development of nephrotoxicity with plasma levels, an alternative suggestion was to adjust dosage on the basis of changes in renal function [10]. In part, the large variations in drug concentration are attributable to differences in concentrations of plasma lipoprotein and a failure to standardize conditions such as temperature under which plasma separation takes place [11]. More recently, the whole blood assay has been developed and obviates the variability of this step. The latter technique is also more attractive since whole blood levels appear to fluctuate less in response to oral or intravenous administration (P. Jacobs, unpublished observation) and might be anticipated to correlate more closely with immunosuppressive events occurring on the surface of other cells such as lymphocytes and monocytes.

In view of the technical difficulties encountered in reproducibly measuring cyclosporin A on samples of frozen whole blood, the technique has been further evaluated, and two practical alternative modifications are reported.

Materials and methods

Blood samples. Venous whole blood was anticoagulated with lithium heparin at a ratio of 140 USE units (U)/10 ml blood
Table 1. Reagents

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer B</th>
<th>Standard solution</th>
<th>Sample solution</th>
<th>Tracer solution</th>
<th>Anti-serum solution</th>
<th>Charcoal suspension</th>
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<tr>
<td>TA</td>
<td>1.2</td>
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</tr>
</tbody>
</table>

- Incubation is for 2 h at ambient temperature, followed by 15 min at 4°C. Separation is by centrifugation after 10 min at 4°C, and thereafter counting of radioactivity in the supernatant.
- TA, total radioactivity in solution; NSB, nonspecific binding equivalent to radioactivity not adsorbed by charcoal in the absence of the antibody; BO, bound 14C tracer at 0 dose of unlabeled cyclosporin A; STD, the standards; and UNK the unknowns.
- The volume can be varied depending upon the anticipated concentration of cyclosporin A in the biological fluids, and volume compensation is effected by adjustment by buffer-B solution. It is imperative that the biological fluid remains the same in all tubes in the assay so that the equivalent adjustments may be made in the standard curve.

(Vacutainer; Becton-Dickinson, Rutherford, New Jersey, USA).

Three separate procedures were followed. First, the well-mixed sample was split and aliquots stored at 4°C and -20°C. Second, a series of dilutions was immediately prepared in buffer at a ratio of 1:50, after which these samples were also stored at 4°C and -20°C. Third, serum or plasma was recovered by centrifugation at 750 g for 15 min (MSE; Super Minor) and stored at -20°C; in one set of experiments, serum or plasma was separated at room temperature (20°C) and, in another experiment, the whole blood sample was equilibrated for 1 h at 37°C, after which the plasma was recovered.

Buffer A. A 0.05-M solution of Tris buffer (Tris [hydroxymethyl] aminomethane hydrochloride) pH 8.5 (Trizma 8.5 Sigma; batch T-5378; St. Louis, MO) was prepared in glass distilled water for each assay.

Buffer B. Tween 20 (Merck; batch 822184; Munchen, FRG) was added to buffer A in a final concentration of 0.03% immediately before each assay.

Control serum. Lyophilized serum (Validate; General Diagnostics, Ireland) was reconstituted with 5 ml glass distilled water for each assay to bring the final protein concentration into the normal range. Alternatively, human AB serum can be used as the control or in the preparation of standard curves.

Control blood. Whole blood was collected from normal volunteers who had not received cyclosporin A, separated, and stored under conditions identical to the test samples. These specimens were used for both control and preparation of standard curves in the whole blood assay.

Tracer solution (cyclosporin RIA kit). A working solution containing 4.5 ng/ml dihydrocyclosporin was prepared by diluting the ethanolic tracer 1:10 with buffer B, supplemented with 10% control serum.

Antiserum (cyclosporin RIA kit). The solution was prepared at least 1 h before use and remained stable at 4°C for four weeks.

Standard dilutions. A total of 1 ml of cyclosporin-A standard containing 40,000 ng/ml was serially diluted, starting at 1 in 20, to give standards containing 2000, 1000, 500, 250, 125, and 62.5 ng/ml. In each case, the plasma or blood used to prepare the standard curves was the same as for the samples being analyzed. Working solutions were prepared from the standards, using buffer B, to give a series of standards containing 4, 2, 1, 0.5, 0.25, and 0.125 ng/0.1 ml.

Sample dilutions. Unless prediluted in buffer and either stored at 4°C or frozen, all specimens of serum, plasma, or whole blood were diluted 1:50 with buffer B for a final concentration of 2 μl of 0.1 ml of buffer.

Charcoal-coated serum. A suspension of 100 ml buffer B containing 0.5 ml of control serum and 1 g of charcoal (Merck; cat. no. 2186, analytical grade; Darmstadt, FRG) was magnetically stirred for 2 h at 4°C immediately before use.

Assay procedure. The standard curve and the unknown samples were run simultaneously in 13 × 85-mm plastic tubes (Medispo, Johannesburg, South Africa), with particular attention to meticulous pipetting technique. All measurements were carried out in duplicate, and reagents were well mixed and allowed to reach ambient temperature before use.

The detailed procedure steps are shown in the assay flow chart (Table 1). Appropriate volumes of buffer, test material, tracer solution, and antiserum were pipetted into the tube, with mixing after each addition, equilibrated at 20°C for 2 h, and then allowed to stabilize in a water bath at 4°C for 15 min. Serum-coated charcoal was added and the tubes agitated on a vortex mixer (Gencos Instruments, cat. no. 34524-200, Breda, The Netherlands). The tubes were then held at 4°C for a further 10 min, after which they were centrifuged at 2000 g for 5 min at 4°C.

A total of 1.0 ml of the clear supernatant was pipetted into counting vials containing 10 ml of scintillation fluid (Instagel, cat. no. 602100; Packard, IL), thoroughly mixed, and radioactivity determined in a liquid scintillation spectrometer (Packard Tri-Carb, model C2423). Results were expressed in either disintegrations per minute or counts per minute, corrected for quenching. The radioactivity of the samples was counted to statistical significance.

Calculation of results. The unknown concentrations are derived from the dose-response standard curves using the unweighted logit-log-linearization method. Since measured radioactivity cannot be related to the amount of unlabeled cyclosporin, the response is expressed as a function of percent relative binding given by the formula:

\[
\% \text{ B/B}_{0} = \frac{\text{dpm of standard or unknown}}{\text{dpm nbs}} - \frac{\text{dpm nbs}}{\text{dpm nbs}} \times 100
\]

Recovery experiments. Analytical grade cyclosporin A in suitable solution added to plasma, serum, and whole blood, whether manipulated at 37°C or 20°C, was quantitatively recovered and showed a pattern indistinguishable from results obtained with the experimental samples, thus excluding interference that might result in underestimating the presence of this material.
Table 2. The effect of separating serum or plasma from red cells at ambient room temperature (20°C) is consistently and significantly lower than when the same procedure is carried out at 37°C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>37°C ng/ml</th>
<th>20°C ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>3</td>
<td>79</td>
<td>63</td>
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<td>4</td>
<td>195</td>
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<td>5</td>
<td>310</td>
<td>88</td>
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<tr>
<td>6</td>
<td>263</td>
<td>140</td>
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<tr>
<td>7</td>
<td>320</td>
<td>255</td>
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<tr>
<td>8</td>
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<tr>
<td>13</td>
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<td>625</td>
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<td>14</td>
<td>1325</td>
<td>1025</td>
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<tr>
<td>15</td>
<td>1975</td>
<td>1925</td>
</tr>
<tr>
<td>16</td>
<td>2510</td>
<td>2125</td>
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<td>17</td>
<td>2525</td>
<td>1962</td>
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<tr>
<td>Mean</td>
<td>844</td>
<td>640</td>
</tr>
<tr>
<td>SD</td>
<td>560</td>
<td>532</td>
</tr>
</tbody>
</table>

Statistical methods. Statistical analyses were carried out using the students' paired t-test [12], coefficient of correlation [13], and significance [14], according to standard methods.

Results

Comparison of serum to plasma

There was no statistically significant difference in cyclosporin-A levels between serum and plasma, provided that the conditions of cell separation were identical.

Effect of temperature

Separation at ambient (20°C) temperature compared with 37°C consistently underestimates the amount of cyclosporin A in serum or plasma by 30% (p < 0.005). This difference can be abolished either by immediate cell separation or allowing the sample to remain for 1 h at 37°C before centrifugation at the same temperature and removal of plasma (Table 2).

Comparison of serum or plasma to whole blood

Over the entire therapeutic range, the levels of cyclosporin A in serum or plasma separated at room temperature were consistently lower than those determined by radioimmunoassay on whole blood that had been stored at 4°C or prediluted with buffer; levels for frozen whole blood were intermediate. The ratios were 361:1041:873 ng/ml (Table 3); the precise numbers are derived by correcting the observed readings for dilution necessary to express results in ng/ml.

When whole blood was frozen and thawed, even permitting temperature to reach stability at 20°C (ambient or room temperature) and then mixing on a vortex shaker to obtain uniform dispersion of the sample, the amount of cyclosporin A was under-

Table 3. At any given dosage schedule, cyclosporin-A levels measured by radioimmunoassay are consistently lower in serum than in whole blood. Furthermore, when blood is retained at 4°C or prediluted with buffer the cyclosporin-A levels are significantly higher than when the sample is frozen, thawed, and then reconstituted.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Serum*</th>
<th>Blood*</th>
<th>Frozen whole blood*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>62</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
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<td>402</td>
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<td>67</td>
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<td>74</td>
<td>838</td>
<td>763</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>399</td>
<td>343</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>563</td>
<td>507</td>
</tr>
<tr>
<td>9</td>
<td>86</td>
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<td>10</td>
<td>91</td>
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<td>215</td>
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<td>863</td>
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<tr>
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<td>1175</td>
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<td>1363</td>
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<td>444</td>
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<td>22</td>
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<td>1800</td>
</tr>
<tr>
<td>30</td>
<td>1163</td>
<td>1825</td>
<td>1675</td>
</tr>
<tr>
<td>Mean (n = 30)</td>
<td>361.67</td>
<td>1045.2</td>
<td>879.6</td>
</tr>
<tr>
<td>SD</td>
<td>347.63</td>
<td>467.3</td>
<td>444.92</td>
</tr>
</tbody>
</table>

* Separated at ambient room temperature (20°C).
* Stored at 4°C, well mixed, and diluted immediately before assay. Predilution with buffer and storage of these aliquots give essentially the same results.
* Anticoagulated whole blood is frozen, thawed, and well mixed before assay.
estimated in comparison to whole blood that had been stored unfrozen (4°C). In parallel studies, blood immediately diluted with buffer and stored at 4°C, or when such prediluted samples were frozen and then thawed, gave essentially similar results. The difference between whole blood that had been frozen and thawed and samples diluted from whole blood stored at 4°C or prediluted with buffer and then frozen was, respectively, 873 ng/ml and 1041 ng/ml. This difference was significant (p < 0.005) and appears to be related to changes produced in the viscosity of frozen whole blood aggravated by the formation of microaggregates that form under these circumstances.

**Stability of samples**

Whole blood stored at 4°C for up to two weeks gave uniform results. Similarly, blood premixed with buffer and stored at 4°C or frozen gave stable assay values that did not differ from measurements carried out on the original sample.

**Quality control**

Reference samples analyzed serially in duplicate gave intraassay and interassay variations of 15%, with a reliability coefficient of 0.997 and 9.3% with a test to retest reliability coefficient of 0.95, respectively. The corresponding figures for whole blood were 6.8%, with a reliability coefficient of 0.996 and 8.3% with a test to retest reliability coefficient of 0.992. None of these differences was statistically significant.

**Comparison of antiserum**

Comparison of radioimmunoassay for serum, plasma, or whole blood, using antiserum raised in rabbits or sheep, shows the latter to be more sensitive, but ultimate results were identical with both antisera.

**Liquid scintillation counting**

Chemiluminescence is a major variable, particularly in the whole blood assay. Two approaches were used to resolve this difficulty. First, radioactivity in samples could be determined directly as disintegrations per minute or the counts per minute corrected for quenching; under these circumstances, both approaches gave comparable values. Alternatively, the samples could be allowed to stand overnight at 4°C or 0.1 ml of 1 N AR grade hydrochloric acid added (British Drug Houses, London, England) and the samples read immediately. This modification abolished chemiluminescence promptly and reliably.

**Discussion**

Cyclosporin-A administration may be associated with deterioration in renal [6, 15–17] and hepatic [7, 18] function. The former occurs with greater frequency and is aggravated by concurrent use of certain antibiotics and diuretics in a seriously ill patient, so that the widest margin of safety requires regular measurement of both blood levels and biochemical profile.

Serum or plasma separated at 37°C for radioimmunoassay of cyclosporin A gives levels variably above those obtained when centrifugation occurs at room temperature when there is a shift in the balance of equilibrium favoring red cells [19–22].

Whole blood radioimmunoassay is advantageous, particularly when separation procedures are difficult to standardize. We have, furthermore, found these levels more stable than simultaneous measurements on plasma and consistently higher for any given dosage schedule. The extent to which red cell findings can be extrapolated to other cells, such as the lymphocytes more directly involved in the immune process, is presently unknown, but reduced incorporation of tritiated thymidine has been demonstrated in the mixed-lymphocyte reaction when trough levels are maintained between 200 and 400 ng/ml and at a peak 4 h after dosing between 500 and 700 ng/ml. Used in this way, less nephrotoxicity has been encountered but, if peak whole blood levels are persistently greater than 1000 ng/ml, biochemical evidence for renal dysfunction with rising serum creatinine and urea, together with decreased clearance values, can be anticipated.

Three additional points require comment. First, the method described for whole blood radioimmunoassay using frozen and then thawed blood is technically difficult and gives variable underestimation of cyclosporin-A concentration. A useful modification is to store the blood at 4°C and then mix well at room temperature before preparing dilution. Alternatively, it is equally acceptable to pipette the appropriate aliquots of well-mixed blood into buffer immediately after collecting the sample; these are then ready for assay and can be stored at either 4°C or −20°C for batch analysis. Samples handled in either of these two ways gave results that were consistently higher than the frozen whole blood method. Second, where liquid scintillation counting equipment does not have the facility for expressing
results in disintegrations per minute or for quench correction, the addition of hydrochloric acid to the counting medium abolishes chemiluminescence and thereby avoids a delay in obtaining reproducible readings on the samples. Third, distribution between red cells and plasma will be affected by hematocrit (21) so that this variable must be taken into account, as with improvements that may follow successful kidney transplantation. In more general terms there is a correlation between whole blood radioimmunoassay and the concentration of parent substance determined by HPLC (23), but because of cross-reactivity with metabolites the radioimmunoassay may overestimate effective immunosuppressive concentrations by a factor of 4–5 (24).

It is concluded that the monitoring of cyclosporin-A levels in clinical practice, particularly where plasma or serum separation is reliable, may be advantageously carried out using the whole blood radioimmunoassay. A practical modification is the storage of anticoagulated whole blood at 4°C rather than by freezing, when subsequent thawing interferes with pipetting. Alternatively, appropriate predilution can be made with buffer immediately after collection of the whole blood and these aliquots stored at 4°C or frozen at −20°C for batch analysis.

Acknowledgments

We thank Jackie Davies for secretarial assistance, Prof. Bob Miller and Dr. Eva Abisch for technical advice, Mr. S. Isacis for help with statistical analysis, Wendy Faulsen for technical assistance, Miss Lucille Wood and Dr. Rob Martell for help with managing the patients and in preparation of the manuscript, and Dr. H.-R. Sanders, Medical Superintendent of Groote Schuur Hospital, for permission to publish. This study was supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the Medical Research Council and National Cancer Association. The cyclosporin A and the radioimmunoassay kits were generously donated by Sandoz Products, Switzerland.

References

Parker JR, Bareford D, Manuel G, Milns B, Hart K, Jacobs P.
The effect of Cyclosporin A and Corynebacterium Parvum on survival of rabbit bone marrow allografts.
SIMULTANEOUS SESSION VII


Chronic GVHD is a late complication of allogeneic marrow transplantation associated with immunosuppression and recurrent infection. To determine the effect of immunosuppressive therapy and prophylactic trimethoprim-sulfamethoxazole (TMP-SMX) on infection acquisition, we reviewed the patients with extensive chronic GVHD transplanted between Jan 1972 and Feb 1982. Since 1977 therapy has included either prednisone alone (Pred, 1.0 mg/kg/day) or in combination with cyclosporine (CS, 1.5 mg/kg/day). Most patients received TMP-SMX (160 mg TMP b.i.d.) during chemotherapy. Since 1980 therapy has been prospectively randomized in a continuing double-blind study of Pred + placebo vs Pred + A2 (Pred +/- A2). Because patients with persistent thrombocytopenia have increased mortality (Blood, 99:673a, 1982), we studied separately those with platelets (plt) > 100,000 at day 100. The following post day 100 infections were analyzed in 198 patients with chronic GVHD (CS aplastic anemia, 147 hematologic malignancy): 1) varicella zoster virus (VZV), 2) non-bacterial interstitial pneumonia (IP), 3) bacterial or fungal pneumonias ("other" pneumonias), 4) bacteremia or meningitis ("other" infections). The table presents the percentage of these infections in the different treatment groups.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Per Cent with Infection</th>
<th>Per Cent with Other Infec</th>
<th>Death Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pred alone</td>
<td>V2</td>
<td>16</td>
<td>55</td>
</tr>
<tr>
<td>* Pred +/- A2</td>
<td>24</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Pred +/- A2</td>
<td>66</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Pred +/- A2</td>
<td>66</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pred alone</td>
<td>V2</td>
<td>16</td>
<td>55</td>
</tr>
<tr>
<td>* Pred +/- A2</td>
<td>24</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Pred +/- A2</td>
<td>66</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Pred +/- A2</td>
<td>66</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>72</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

The number of infectious episodes determined in patients receiving TMP-SMX and expressed as a ratio: pneumocystis (0/6) / IP, pneumococcal pneumonia (4/17), and pneumococcal bacteremia/meningitis (1/22).

A Log rank analysis of time to first infection showed that recipients of TMP-SMX prophylaxis had fewer non-V2 infections than recipients of Pred +/- A2 (p < 0.001). This difference remains statistically significant when adjusted for the year of transplant and suggests an important role of TMP-SMX prophylaxis in patients with extensive chronic GVHD.

X MARKS THE SPOT: TREATMENT WITH CYCLOSPORIN A IN ALLOGENEIC BONE MARROW TRANSPLANTATION. Jacobs, P., Department of Haematology, Research Centre, University of Cape Town Medical School, Anzio Road, Observatory, 7925, Cape Town, South Africa.

Graft-versus-host disease (GVHD) remains a formidable barrier to the widespread use of allogeneic bone marrow transplantation. This immunologic phenomenon, which may be acute or chronic, occurs despite meticulous matching of donor and recipient at the genetic level. Attempts to prevent or abrogate these syndromes have met with limited success, including the recent introduction of Cyclosporin A (CyA) as an immunosuppressive agent. Although different regimens have been tested in the recipient no attention has been given to pretreatment of the donor with CyA. Since it is theoretically possible that immunocompetent lymphocytes transferred to the recipient may participate in the pathogenesis of GVHD we tested the possibility that administration of this unique fungal metabolite to both donor and recipient might decrease the expression of acute or chronic GVHD. Forty-one patients underwent allografting from HLA-identical and mixed lymphocyte non-reactive siblings. Group I (n=24) were transplanted before CyA was available and severe GVHD occurred in 12 patients (40%) during the acute stage and in 7 (22%) during the chronic stage. Attempts to treat these patients with additional corticosteroids or to use different regimens resulted in severe GVHD in 3 (37.5%) of the patients. In Group II (n=9) the acute stage of GVHD was treated before CyA was available and in 4 (44%) of the patients severe GVHD occurred in 2 (22%). Attempts to treat these patients with additional corticosteroids or to use different regimens resulted in severe GVHD in 3 (37.5%) of the patients. In Group III (n=9) the overall response rate of CyA was 5 (55.5%) of the patients with prompt response to corticosteroids in 3 and complete remission in 2. In Group IV (n=11) the overall response rate of CyA was 7 (63.6%) of the patients with prompt response to corticosteroids in 3 and complete remission in 4. In Group V (n=11) severe GVHD occurred in 2 (18.2%) of the patients with prompt response to corticosteroids in 2. In Group VI (n=11) severe GVHD occurred in 2 (18.2%) of the patients with prompt response to corticosteroids in 2. All studies are carried out with fully informed consent and plasma CyA levels carefully controlled using routine assays. No donor toxicity was encountered although reversible renal dysfunction was present in over 40% of recipients. These preliminary data suggest that a short period of donor pretreatment with CyA may further reduce the incidence and severity of both acute and chronic GVHD compared to our previous study.
1983 ANNUAL MEETING ABSTRACTS
Twelfth Annual Meeting

INTERNATIONAL SOCIETY
for
EXPERIMENTAL HEMATOLOGY

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Kensington Exhibition Centre & Kensington Close Hotel
London, England, The United Kingdom

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The Royal Postgraduate Medical School
London, England
The United Kingdom
10
Cyclosporin A
Current Status. Including the Cape Town Experience

Peter Jacobs  The University of Cape Town Medical School, and Groote Schuur Hospital, Observatory, Cape Town, South Africa
Linda Eglin  The University of Cape Town Medical School, Cape Town, South Africa

I. INTRODUCTION

The well-being of the human race is constantly challenged by the presence of antigens in the environment; some may be foreign or extrinsic, such as bacteria or viruses, while others are intrinsic, being known as autoantigens. Although superficially different, the physiological response in each situation is linked by the functional integrity of the immune system. Historically, credit is due to the stimulating work of early immunobiologists, including Burnet (1), Billingham et al. (2), and Medawar (3), but it is the vast amount of work carried out in the last decade that forms the basis for understanding the physiology and pathology of the immune system (4,5). Under normal circumstances, the immunological response differs between antigen that is foreign, or non-self, and that which belongs, or self. The former elicits the activation of highly specific humoral and cell-mediated mechanisms which interact to remove the antigen, often involving a nonspecific immune process such as production of opsonins and phagocytosis. In contrast, the development of immune tolerance prevents the initiation of a similar process directed against host antigen.

The complex nature of the immune system is gradually being unraveled and disturbances, congenital or acquired, in the humoral or cellular components are being increasingly correlated with distinct clinical syndromes. Thus, failure of immune surveillance to distinguish self from non-self may lead to an autoimmune reaction exemplified by systemic lupus erythematosus. In addition, there is a whole new discipline developing in medicine in which foreign tissue is transferred from one individual to another, and in this process of transplantation, graft acceptance and subsequent function often depend upon the deliberate suppression of the immune response.
In parallel with the better understanding of the structure and function of the immune system and the disturbances that give rise to clinically important disease entities (5), manipulation of immune responsiveness has become the goal of therapy in many patients. Not surprisingly, there has followed the development of a large number of drugs and other agents whose effects vary from stimulation through modulation to immunosuppression. It is the latter that are of particular interest since they are most widely used in the treatment of autoimmune disease and are indispensable in tissue transplantation.

A. Immunosuppressive Drugs: Current Shortcomings

Agents for the suppression of immunologically mediated inflammatory processes are, as a group, nonselective. As a result, undesirable side effects are evident on practically every organ and tissue in the body, notably, the gastrointestinal tract and the hematopoietic system. For this reason, the ever present challenge is for the development of substances with clearly defined sites of action. In theory, these could be directed against the non-specific immune process, interfering with phagocytosis of particulate antigen. Alternatively, the action could be restricted to specific or adaptive immunity influencing macrophages or different lymphocyte subpopulations.

Initially, drugs interfering with immune phenomena included the antihistaminics (6), the anti-inflammatory drugs (7), and the immunosuppressive agents themselves (8). Not only are the actions of these drugs still awaiting accurate characterization, but many have wide-ranging complications, including myelotoxicity (9), and their lack of specificity also predisposes the patient to the serious hazards of opportunistic infection (10) and the risk of malignant disease (11).

In view of these shortcomings, attention has increasingly been directed at improving specificity. The first step in this direction was the development and then the use in clinical practice of antilymphocyte globulin (12). The latter approach suffers from limitation in that there is relative lack of specificity since large numbers of lymphocyte subpopulations may be removed or their function destroyed. Subsequently, antithymocyte globulin (13) was developed in the hope that the target population could be narrowed to T lymphocytes. Further refinement has been the veritable explosion of monoclonal antibodies that followed the introduction of the hybridoma technique (14). While these biological reagents are becoming more abundant, and already whole libraries exist, their availability for therapy is still limited.

It is in this context that the unique and interesting antilymphocyte agent, cyclosporin A, needs to be judged.

B. Cyclosporin A: Historical Perspective

In the general quest to develop greater selectivity for immunosuppressive agents, one option is to identify those with an action predominantly against a specific lymphocyte subpopulation. Cyclosporin A meets this criterion, since the fungal metabolite appears to modify T-helper activity. Of special note is a surprising lack of bone marrow toxicity so that it already has an important role in clinical programs while, at the same time, attracting attention in the study of basic immunological phenomena.

Historically, the synthesis of the cyclosporins began with the isolation of a metabolite from a new strain of Trichoderma, one of the fungi imperfecti. In practice, the large-scale production is achieved by growing the fungi in submerged culture and then extracting the cyclosporin polypeptides from the mycelia since they are not released into the culture medium.
Based on this fermentation procedure, a series of developments took place in the laboratories of Sandoz in Switzerland and, while credit has been given to all those involved (15), Jean Borel stands out as the driving force in getting the product now known as cyclosporin A to clinical trial. It is interesting that the fungal extract has little fungistatic activity in vivo, but it was known that such metabolites of microbial origin may possess cytostatic or other pharmacological activities. Efforts were then made to define its properties, and particular attention was given to elucidating any immune suppressive effects (16,17).

In a long series of systematic studies, this latter property was compared with a variety of immunosuppressive, cytostatic, and other reference drugs, using several different models. These initial observations revealed two important properties of cyclosporin A, in that the immunosuppression was separable from more general cytotoxic side effects and that both humoral and cell-mediated pathways were affected (16,17).

Following purification, characterization by x-ray analysis (18,19), and synthesis, the pure product was further tested. Comparing murine splenic and mastocytoma cells, an interesting phenomenon was demonstrated (20): cyclosporin A, like hydrocortisone and antilymphocyte serum, had a selectivity for lymphoid cells, and this was in sharp contrast to conventional cytostatic drugs which, in this assay system, inhibited both cell types to a similar degree. Furthermore, the effect with the fungal metabolite was achieved without the usually associated myelotoxicity that characterized administration of equipotent doses of immunosuppressive drugs such as azathioprine. Further studies by Borel and his associates (21,22) showed that cyclosporin A was not lymphocytotoxic and had selectivity for the T-helper cells; an effect was also demonstrable on T-effector cells, but this was less evident and depended on the model used. Last, these actions were primarily evident during the induction phase of lymphoid cell proliferation and not during mitosis.

Although initially bedeviled by problems with absorption, the group at Cambridge (23-26) confirmed the claims for an immunosuppressive effect, using an animal heterotopic cardiac allograft model. Subsequently, similar series were reported in patients with mismatched cadaver kidney transplants (27) and following allogeneic bone marrow transplantation (28).

Clearly, then, cyclosporin A is a unique and potentially important new immunosuppressive agent. However, while it enjoys current widespread interest, there remain a number of important questions to be answered. What, for example, is its exact mechanism of action? In which body compartment, serum or red cells, should concentrations be measured? What minimal level in the plasma reflects optimal immunosuppression? Can peak and trough measurements in the serum, whether determined by radiolimunnoassay or high-performance liquid chromatography, be used to monitor the development of short- or long-term toxicity? Is there a particular place for a natural killer cell assay in guiding therapy? What is the ultimate role of cyclosporin A in chronic inflammatory disease and organ transplantation? Should it be used as a single agent or in combination? If the latter, what combination and on what schedule?

Undoubtedly, we are on the threshold of a change in direction for the use of immunosuppressive agents. In this context, cyclosporin A represents an advance in specificity on antilymphocyte globulin comparable with the way in which that material, whatever its inherent limitations are, was a step forward from the use of the cytotoxic drugs where myelotoxicity often limited adequate therapy.

To place in perspective what is currently known about cyclosporin A, we have reviewed the available information and, where relevant, integrated new data from our own experience.
II. CHEMISTRY

A. Origin

Cyclosporin A is a fungal cyclic undecapeptide $C_{62}H_{111}O_{12}$, isolated from *Tolypocladium inflatum Gams*.

B. Structure (Fig. 1)

The compound has several N-methylated amino acids and one novel C$_9$-amino acid. It has a molecular weight of 1202.6 daltons, and its structure is shown in Fig. 1 (18, 19, 29).

C. Properties

The peptide is neutral, rich in hydrophobic amino acids, insoluble in water and a-hexane, but very soluble in all other organic solvents and lipids. Cyclosporin A may be crystallized to a white hydrophobic powder, and this property has given rise to some of the difficulties in its preparation for clinical use.

Originally, animal experiments were carried out, suspending the active principal in 0.5% tragacanth, but more recently a solution in ethanol and Tween has been developed.

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Figure 1 The chemical structure of cyclosporin A. The molecule is thought to exist partly as an open loop and partly as a $\beta$-pleated sheet. The $\beta$-hydroxy group in the C$_9$-ENE side chain forms a hydrogen bond with the carbonyl oxygen atom of the same amino acid and may account for the immunosuppressive activity of this fungal metabolite. (From Ref. 19.)
Both preparations are well tolerated by experimental animals and when given, using an olive-tipped gastric cannula, dependable administration schedules are easily possible. In clinical trials, a recently formulated drinking solution is well tolerated, particularly in a taste-disguising milk solution to which chocolate, cocoa, or Caotina has been added.

Injectable forms are available, prepared by dissolving cyclosporin A in a mixture containing ethanol and Miglyol, and subcutaneous, intramuscular, and intravenous routes in both experimental animals and patients, in our experience, have been used without any local side effects.

D. Assay Methods

1. High-Performance Liquid Chromatography (HPLC)

The determination of cyclosporin A (OL 27-400) is possible in human plasma and urine, using high-performance chromatographic separation in a reversed phase mode and ultraviolet absorption detection at 210 nm. At this wavelength, the compound has an extinction epsilon of 45,000 with an \( \epsilon_{\text{max}} \) of 66,000 at 195 nm in methanol. The internal standard used is cyclosporin D (33-804), differing only slightly from cyclosporin A in structure and chromatographic properties. The detection limit is 20 ng/ml, and the method has a linear calibration curve in the concentration range of 25-2000 ng/ml. Precision is good, and metabolites do not interfere with determination of the parent compound so that this is a reliable technique with high specificity (30,31).

This method has been further examined and its sensitivity confirmed (32). These workers have emphasized the higher levels obtained with radioimmunoassay when compared with HPLC, which may be attributed to cross reactivity of the antibody with metabolites of the cyclosporin. The generally poor correlation between the level of cyclosporin A in the plasma when determined by the radioimmunoassay and development of toxicity emphasizes a role for HPLC as a means to monitor the parent compound. Similarly, this technique provides the only method for measuring metabolites in the various compartments that include blood cells, biopsy material, and from detailed postmortem studies.

2. Radioimmunoassay (RIA)

Cyclosporin A (OL 27-400) can be determined by radioimmunoassay, using an antiserum raised in rabbits to cyclosporin C (Therovil) hemisuccinate coupled to free amino acid groups of guinea pig immunoglobulin (IgG). The antiserum reacts primarily with cyclosporin A and cross reacts with the metabolites, dihydrocyclosporin A, and dihydrocyclosporin C (33,34).

Based on the continuous use and development of this assay in our laboratory, we have found the standard curve to be reproducible between 0.125 and 4 ng/tube, with linearity from 0.50 to 4 ng/tube, the assay to be sensitive to a lower limit of 62.5 ng/ml, with an interassay variation of less than 5%.

We have not personally evaluated the assay for cross reactivity with metabolites, although this has been reported (33,35). Abish (33) has confirmed the specificity of the assay and tested metabolites designated 1, 8, 17, and 21 for cross reactivity with the antiserum 031180. The highest concentration for these compounds was 40.5, 122, 8, and 64 ng/0.1 ml, respectively. Cross reaction with cyclosporin C and dihydrocyclosporin C is 100%, because cyclosporin C was the hapten used to raise the antibodies.
The detection range with 2-μl samples is 40-2000 ng/ml plasma: but 10 μl of unknown plasma is needed to detect between 10 and 400 ng with any precision.

We consider the radioimmunoassay a dependable method for determining the parent substance and its metabolites in the serum and urine of experimental animals and in patients on clinical trial. It is, however, necessary to appreciate its limitations which are largely the result of cross reaction with metabolites. The question of distribution between the different cellular components in blood and the plasma phase is another theoretical consideration in using RIA.

3. Correlation and Perspective between HPLC and RIA

The correlation between high-performance liquid chromatography and radioimmunoassay in serum (36) has been examined in patients following allogeneic bone marrow transplantation (37), and the results compared by a linear regression analysis. In 50 observations over a 35-day period with the patients on oral administration, the coefficient of correlation was high (r = .933), with the value determined by RIA being 30-70% higher due to cross reacting metabolites.

These data confirm not only the reliability of both methods but establish their linear relationship. Nevertheless, the role of the two methods in monitoring clinical trials requires further attention, particularly since difficulties exist with inability to discriminate between therapeutic administration with optimal immunosuppression and inadequate or excessive treatment (35).

4. Cyclosporin A in Serum and Other Tissues

The distribution and binding to blood components have been reviewed (38), and in vitro studies have shown uptake predominantly by lipoproteins and erythrocytes. The lipoproteins, obtained by ultracentrifugation and fractionation, showed that over 60% of the drug was associated with this compartment independently of drug concentration. Uptake by erythrocytes was rapid and linear, accounting for 50% of the uptake by blood cells. In contrast, leukocytes bound only between 10 and 20%, but the kinetics of this phenomenon were nonlinear and the process was saturable.

Very little data are available at the present time on the distribution of cyclosporin A or its metabolites in different cellular compartments in the body, and a major deficit in our current knowledge is whether there is preferential binding, perhaps to lymphocytes, monocytes, or early hematopoietic precursors in the marrow, that may have profound immunological effects and where pharmacological monitoring by means of plasma levels may be inappropriate.

It may therefore be concluded, with regard to the assay methods currently available, that while radioimmunoassay is suitable for determining plasma levels in experimental animals and clinical studies, a great deal more essential information is required. Of particular importance will be the correlation of immunological effects with levels in the different tissue compartments and clarification on whether these effects and such toxicity as occurs correlate most closely with determination of parent substance or the total spectrum of metabolites determined immunologically by radioimmunoassay.

III. IN VITRO STUDIES

Current interest in the clinical use of cyclosporin A centers on its activity as a potent immunosuppressive agent. The available evidence suggests that these effects are mediated
with little or no bone marrow toxicity. For these reasons it is logical to review first what has been established about the effects that this novel antifungal metabolite exerts on lymphoid cells in vitro. In reviewing what is rapidly becoming a voluminous literature, it is clear that results may, in some studies, have been affected by technical variations. The most notable of these is that since cyclosporin A is an extremely hydrophobic substance, its solubility is critical in standardizing preparation of material for in vitro studies. At the present time, it would seem prudent to follow in minute detail recommendations for its preparation (22). Furthermore, in all studies appropriate solvent control must be included.

A. Nucleic Acid Synthesis

The biological effects of cyclosporin A are its specificity for lymphocytes, a critical dependency on time of administration, and suppression of both humoral and cellular immunity in the laboratory animals (39). The further exploration of these effects (22) was undertaken in vitro in both mouse spleen cells and human peripheral blood lymphocytes. Both the incorporation of tritiated thymidine into DNA and the uptake of radiolabeled uridine into RNA were inhibited by cyclosporin A in doses between 30 and 1000 ng/ml, which are in the range currently achieved in many therapeutic trials. This phenomenon was critically time dependent, requiring addition of cyclosporin A at the same time as the mitogens, concanavalin A or phytohemagglutinin; the later addition of the cyclosporin A to the culture failed to have any inhibitory effect. Furthermore, and of note, was that this phenomenon was reversible, not easily by washing, but by elution over a 24-hr period. This reversibility, occurring without loss of cells in the culture, established a lack of lymphotoxicity (26,40).

These results show a primary effect of this agent directed toward early cellular events in which lymphocytes, in response to mitogenic stimulation, are prevented from undergoing blastogenesis. The cells do not lose their proliferative ability since, following elution of cyclosporin A, this function is again evident. It appears likely that the lymphoid cells are arrested in the G0-phase of the cell cycle and that this is a physiological phenomenon explaining the reversibility of this effect.

B. Effect on Lymphocyte Subpopulations

The mixed lymphocyte reaction (MLR) and cell-mediated lymphocytotoxicity (CML) have been used in the study of porcine, canine, and human lymphocytes.

In the pig studies (26), in vitro assay of T-cell proliferation and mixed lymphocyte reaction demonstrates a dose-dependent inhibition in the presence of cyclosporin A.

In dogs (41), the results from the MLR indicate that cells reactive to histocompatibility antigens persist but are suppressed in the presence of cyclosporin A, whereas recognition of foreign antigen occurred predictably in the absence of cyclosporin A in the culture system under controlled circumstances. Similarly, a suppressive effect could be demonstrated when cultures were carried out in the serum from dogs treated with cyclosporin A, with the effect evident as early as 1 hr after oral administration to the animal. Of note was the persistence of the suppressor effect, in two dogs, for as long as 10-12 days after cyclosporin A administration. CML demonstrated the ability of lymphocytes from cyclosporin-treated dogs to generate cytotoxic effector cells against suitable target cells when the assay was carried out in normal pooled serum, but when the cultures were performed in the serum from cyclosporin-A-treated dogs, this cytotoxicity was suppressed completely. These results suggest that the effects do not occur through either
clonal deletion or a suppressor cell mechanism, but that the action is abrogated or suppressed in the presence of cyclosporin A.

The results of MLR and CML assays carried out on lymphocytes derived from human subjects provide support for the animal data (42,43). Thus, cyclosporin A does not favor generation or induction of suppressor cell lymphocytes; it spares them while inhibiting other subpopulations, resulting in an imbalance in the immune system with consequential profound immunosuppression. This phenomenon may result from the inhibition or killing of T-helper subpopulations. Cyclosporin A interferes with memory cell recognition on a short-term basis, reflected in disturbances in the recognition phase of the mixed lymphocyte reaction. In vitro human lymphocyte responses studied in the MLR showed that the induction of cytotoxic or cytolytic lymphocytes was markedly suppressed by minimal amounts of cyclosporin A, whereas that of alloantigen-activated suppressor cells was much less inhibited, results favoring the induction of suppressor cell mechanisms as opposed to cytolytic effector cells in the primary MLR (44). The latter investigators suggest that a state of specific operational tolerance may be induced in vitro which accords with their earlier in vivo data (45). This is thought to occur without clonal elimination, and this altered immunological state was considered to be induced or maintained by an active suppressor cell which could be identified by its adherence to nylon wool.

C. Molecular Basis of Cyclosporin A Activity

A review of the data reported for the in vitro effects of cyclosporin A in animals and in humans does not support clonal deletion, and while opinion is not uniform on the generation or induction of suppressor cell lymphocytes, the profound immunosuppression that this novel agent has appeared to center on T-helper subpopulations (22,42) and is reflected ultimately in diminished activity of cytolytic or effector lymphocytes.

Studies in mice (22) showed that cyclosporin A effectively inhibited plaque-forming cells when assayed using the Mishell and Dutton technique (46). These results provide evidence of an antithelper cell action for this agent. The selective effect on functional B-cell subsets (47) showed that T-dependent and certain T-independent antigens, the TI-1 mitogens, the TI-2 agents, are exquisitely sensitive. These studies emphasize the value of cyclosporin A in analyzing lymphocyte subpopulations, but it is uncertain whether these observations have clinical relevance. If any extrapolation is possible and B and T cells from humans respond in a similar way to mouse cells, then cyclosporin A should have little impact on established antibody responses and therefore be of limited use for suppression of autoantibody production. Furthermore, cyclosporin A should inhibit some primary antibody responses but leave others unaffected, as might occur with bacterial infection.

The central role played by T lymphocytes raised the theoretical consideration that the various observations could be united through an effect on the interleukins or factors that modulate the activation of lymphocytes. In this regard, it has been shown (48) that concanavalin A-activated suppressor T cells respond to T-cell growth-factor stimulation and exert their suppressor function by absorbing this material, with a consequent decrease in its concentration.

The molecular basis for this action of cyclosporin A on T-cell proliferation is explicable by the interleukins which provide communication between different populations of leukocytes. There are two molecules designated Interleukin 1 and Interleukin 2. The first is a monokine, generated at the time antigen is processed by the Ia+ macrophage and has an effect on the T-helper cell. The latter, simultaneously in all probability, senses
the first signal for antigen presentation from the macrophage. The net result of the presentation of processed antigen to the T-helper cell as well as with the second signal from Interleukin 1, is to permit differentiation of the immature helper T-cell with the subsequent release of Interleukin 2 (Fig. 2) (49). Interleukin 2 facilitates further interaction between the antigen bearing cell and lymphocytes with the proliferation of suppressor and cytotoxic T lymphocytes.

Based on this model, researchers (50,51) have presented evidence that cyclosporin A may interfere with the release of Interleukin 1 from macrophages and the release of Interleukin 2 from activated T-helper cells. A word of caution is necessary since the in vivo or physiological significance of these molecules has not yet been clearly defined, but the hypothesis remains an attractive one and compatible with data previously reported by Borel and his co-workers (20,22). It is also not clear what importance must be attached to reports that cyclosporin A inhibits migration-inhibition factor (52), macrophage-activating factor, and other lymphokines (53).

Cyclosporin A has also been studied in vitro on a number of isolated lymphoid cell populations. In general terms, a direct effect is less clearly demonstrable on the B lymphocytes (20). In vitro, cyclosporin A inhibits immunoglobulin secretion in response to pokeweed mitogen-induced B-cell activation. However, this phenomenon is T-cell dependent (54), and its inhibition may be the result of T-cell inactivation. Similarly, it appears that cyclosporin A specifically deletes or at least inactivates the cytotoxic T-cells normally responsible for the in vitro regression of Epstein-Barr virus lines, so that it may be reasonably speculated that the emergence of lymphoma in patients receiving renal allografts may occur on this basis. There is, however, no evidence that this immunosuppressive agent, in its own right, causes lymphoma.

Figure 2. Putative scheme for the release of Interleukin 1 and Interleukin 2, based on the two-signal concept. (From Ref. 49.)
It has also been shown (55) that cyclosporin A will suppress both T- and B-lymphocyte blastogenesis, and the data have been interpreted by these workers as reflecting immunosuppression due to a direct effect on the blast cell rather than being mediated via a third-party accessory cell. This is an interesting study and provides some support for the controversial “decloning” hypothesis suggested as a possible mode of action for cyclosporin A in vivo (56,57). This evidence would suggest that, at least in humans, cyclosporin A is capable of directly inhibiting certain B-cell activation mechanisms, irrespective of whether these are normally dependent or independent of T-cell activation.

Of interest are the effects that cyclosporin A may have on killer cells, and here the evidence remains debatable. In assays using human lymphocytes in mixed lymphocyte culture, cyclosporin A was shown to efficiently prevent the development of cytotoxic T cells directed against target cells expressing the phenotype of the stimulator cells. This occurred without preventing an increase in the natural killing (NK) activity measured against tumor targets (58). In contrast, cyclosporin A has been shown to inhibit human natural killer activity (59), while another study (60) has shown inhibition of natural killing by cyclosporin A to be rapid, dose dependent, and not requiring the presence of T cells, B cells, or macrophages. These workers interpret the data as direct depression of NK activity with cyclosporin A and could find no evidence that this was mediated through suppressor cells.

These effects occur without marked reduction in lymphoid tissue, notably the spleen, and are in contrast to the effects of other immunosuppressive agents such as cyclophosphamide and azathioprine. The findings are consistent with cyclosporin A producing its effect by interference with stimulation or proliferation of lymphoid cells in the spleen. Similarly, for the thymus (61), data show that the immunosuppressive effects produced by cyclosporin A in vivo are not mediated by the pituitary-adrenal gland system and the results are, therefore, in line with those found in mesenteric lymph nodes and spleen.

What conclusions may be drawn from all this data? The in vitro effects of cyclosporin A are the basis for understanding the observations both in experimental models and in clinical trials. Although results from many studies are not uniform, a number of conclusions are possible. Cyclosporin A acts at a very early stage in the immunologically mediated response to foreign antigens. The available evidence suggests that the major action is on T-lymphocyte-mediated help. The molecular basis is most likely to be by blocking the release of Interleukin 1 from macrophages and thereby preventing the release of Interleukin 2 from T-helper lymphocytes so that generation of cytotoxic T-lymphocytes does not take place. In addition, evidence is accumulating that cyclosporin A has a direct effect both on cytotoxic T and natural killer cells. Its direct action on B cells, if any, awaits clarification.

D. Effects on Nonlymphoid Cells
1. In Vitro Marrow Culture

The most striking observation to be made about in vitro assessment of cyclosporin A on both hematopoiesis and lymphocytopoiesis, using culture techniques, is the paucity of data on which to base any reasonable assessment. Myelopoiesis in humans has been assessed, using an in vitro culture technique (62,63). These studies have limited relevance for the clinical situation since doses between 5 and
10 μg/ml were studied, whereas in therapeutic terms, doses around 1 μg would be much more realistic. Nevertheless, a clearcut reduction in granulocyte-macrophage: colony-forming units in culture (GM:CFUc) was observed, being most obvious at the higher level. In the data reported by Gordon and Singer (63), the T lymphocytes were affected much more than B lymphocytes or hematopoietic precursor cells in the range of 1-5 μg/ml.

Our experience differs from this since the in vivo situation was more closely approximated by using serum from patients containing between 500 and 1000 ng/ml. Control studies were run in parallel using stored serum from the same patients before starting cyclosporin A administration. There was a clearcut uniform suppression of erythroid colony formation, but granulocyte and macrophage growth was normal. These findings, furthermore, correlated with reduced erythropoiesis in the marrow, reflected in low reticulocyte counts and persisting mild anemia while granulopoiesis and megakaryopoiesis were apparently normal. Confirmatory experiments are in progress.

In studies carried out on T-lymphocyte colony formation from peripheral blood and bone marrow (64), it was shown that the effect was most marked on peripheral blood lymphocytes with bone marrow cells being less sensitive, suggesting that this may contribute to the role of this agent in treating graft-versus-host disease in humans.

2. Peripheral Blood Hematology

In mice (39), comparison of cyclosporin A with other immunosuppressive agents showed much less leukopenia, although lymphocytes were approximately 20% below control values. Platelets were not affected. Evaluation of changes in the peripheral blood are difficult to interpret since, for the most part, this immunosuppressive agent is used in patients undergoing allogeneic bone marrow transplantation, of which most are already pancytopenic. However, in a group of patients receiving cyclosporin A as the primary form of treatment for severe acute aplastic anemia, where there was no transplantation option, we have been unable to document any significant changes in peripheral blood hematology.

3. The Phagocytic System

In vitro studies on phagocytic cells (55) failed to show any effect, suggesting that immuno-competent lymphocytes are affected rather than the adherent, radiation-resistant, labelling accessory population.

Similarly, in serial studies on patients following transplantation or those with marrow aplasia receiving cyclosporin A as a primary form of therapy, we have demonstrated no effect on random migration, chemotaxis, phagocytosis, and intracellular killing.

4. Malignant Cells

Tumor cells studied in vitro are of interest. Available results show that the major activity in all the assay systems tested appears directed at T-cell tumors with very little effect on nonlymphoid lines. The common factor appears to be the presence of recognizable T-cell-associated surface antigens, and on this basis cyclosporin A would appear to selectively recognize a series of differentiation antigens on lymphoid cells and their malignant counterparts. It is of further interest that inhibition of permanently growing cell lines requires much higher concentrations than are necessary to produce similar quantitative effects in mitogen-induced T-cell proliferation.
IV. PHARMACOLOGY

A. Human

A single 600-mg oral dose of drinking solution of cyclosporin A, equivalent to approximately 10 mg/kg lean body mass, has a maximal plasma concentration between 242 and 1246 ng/ml when determined by high-performance liquid chromatography. This peak is reached in 3-4 hr after administration and is characterized by a lag time of 0.65 hr, after which oral absorption is fairly rapid with a half-life of about 1 hr. There are two recognizable disposition phases with an α component having a half-life of 1.2 hr and the β clearance curve having a half-life of 27 hr. The maximum concentration is reached in the peripheral compartment after 8 hr; within 12 hr there is roughly eight times more cyclosporin A in this pool than was present in the blood (65).

In a further series of studies reported by the same authors, nine patients undergoing allogeneic bone marrow transplantation had serum levels of cyclosporin A determined after initial intramuscular or continuous intravenous infusion at total doses of 20 mg/kg/day, followed by maintenance at a dose of 12.5 mg/kg/day. These patients showed similar kinetics to the six patients who received a single 600-mg oral dose (65).

In a series of eight studies (66), the clearance of cyclosporin A from plasma was measured, using radioimmunoassay (Fig. 3) and a maximum concentration was found between 2 and 4 hr, with the peak at 2 hr. All patients received oral cyclosporin A as a drinking solution. These studies provide information only for absorption and clearance from the plasma.

The pharmacology is best explained by a two-compartment open model (65) which proposes that cyclosporin A, which is lipophilic, will leave the plasma and distribute itself widely in the tissues of the body, collectively designated as the peripheral compartment. While the validity of this hypothesis awaits confirmation, it has the attraction of emphasizing the need to measure cyclosporin A in tissues other than just the plasma. Our own studies (66) confirm reports that red cell or whole blood levels may be higher than those found in plasma (67). The exact relationship between cyclosporin A levels determined by radioimmunoassay for serum and whole blood hemolysate remains to be established. In this regard, it is theoretically possible that the immunosuppressive level needed may be reflected best by whole blood concentration of cyclosporin A; in contrast, monitoring of plasma concentration may provide a basis for avoiding dose-dependent and reversible renal dysfunction.

The detailed metabolism of cyclosporin A is unknown. However, in pooled plasma collected between 2 and 8 hr after patients had received cyclosporin A in olive oil, 27% of the radioactivity remained present as the parent drug at concentrations of 210 ng/ml. Of importance was the demonstration that 12 separate radioactive components could also be detected, but none was considered to be of major importance. Furthermore, the mean cumulative urinary excretion of radioactivity in a 96-hr period corresponded to 6% of the original dose together with 15 radioactive analogs. These data demonstrate that $^3$H-OL 27-400 is rapidly and extensively metabolized in man to provide a qualitatively similar pattern of metabolites in both plasma and urine.

Further studies on the distribution and binding of $^3$H-OL 27-400 show that over 60% of the drug is associated with lipoproteins and that this distribution occurs independently of drug concentration. The second major blood component that takes up the cyclosporin A are the erythrocytes. At plasma concentrations between 25 and 500 ng/ml, this population will account for approximately 50% of cellular binding and shows a linear
Figure 3  Mean plasma level from eight studies, showing peak at 2 hr. (From Ref. 66.)
distribution with dosage. The leukocyte accumulation varies between 10 and 20% and appears to be a saturable process. The importance of this compartment may be underestimated if only binding capacity is considered, but it may play an important role if there is differential distribution between the various leukocytes. Thus, should the major concentration occur in monocytes and lymphocytes, a greater effect on the immune process becomes a theoretical consideration.

It is this distribution pattern between the lipoproteins in the plasma compartment and the cells, notably the erythrocytes, that raises the important question of the best material for assay to monitor cyclosporin A doses. One approach would be to use radioimmunoassay to detect all immunologically related analogs and to avoid underestimating total body content by measuring levels in whole blood hemolysate.

B. Experimental Animals

Pharmacokinetic studies, including biotransformation, have been carried out in rat, dog, and monkey. In these experiments, tritium-labeled drug was used to examine absorption, distribution, and excretion characteristics, and the data are based on partitioning of total radioactivity.

In rats, absorption was low (12%) when given orally in a solution of Myglyol, with the drug eliminated mainly by the biliary route. Both oral and intravenous administration levels of radioactivity were higher in the tissue of peripheral compartment than in the blood, suggesting tissue affinity for the unchanged drug or its metabolites.

In dogs, oral absorption from a Myglyol solution was again poor, but reached 50% when administered in olive oil, with peak levels between 2 and 3 hr. Biotransformation was extensive, with only trace amounts of parent drug being detectable in the urine.

In rhesus monkeys, absorption characteristics were essentially the same as in dogs, with about 20% of the drug excreted by the kidneys and the biliary route accounting for the majority.

In rabbits, absorption was poor from polyethylene glycol. The presence of a major metabolite and absence of parent drug in the urine were evidence in favor of extensive biotransformation.

In our rabbit studies, we were able to confirm the poor absorption of cyclosporin A when given as the drinking solution. Even on a twice-daily schedule with doses studied systematically between 50 and 300 mg/kg, there was a relatively poor correlation between intake and plasma levels. Using radioimmunoassay, it was seldom possible to achieve consistent concentrations in the plasma in excess of 500 ng/ml. Furthermore, both time- and dose-response curves showed a peak for the rabbit between 1½ and 2 hr (Fig. 4).

The isolation and characterization of the metabolites have been extensively studied (67). The isolation was undertaken on DEAE A25 Sephadex and chromatography on amberlite XAD-2 with the final extraction into diethyl ether. The crude components were purified by liquid chromatography and defined spectrascopically and by amino-acid analysis. The four major metabolites which were designated 1, 8, 17, and 21 contain the intact oligopeptide structure of cyclosporin A, with minor modifications resulting from hydroxylation and N-demethylation reactions. Analysis of all the available information on the metabolism of cyclosporin A indicates that the main biotransformation takes place along a pathway leading to the production of one polar, water-soluble, metabolite designated peak 1, which has not yet been definitively characterized, and a number of associated metabolites extractable with organic solvents such as diethyl ether.
**Figure 4** Rabbit studies showing relationship between peak levels and time at different oral doses of cyclosporin A. The dose approximation in serum levels between the 100 and 150 kg/mg/day curves suggests that an absorption maximum may operate for oral cyclosporin A administration. (From Ref. 66.)

In the light of this information, it should be realized that the pharmacokinetic data which are based on radioactive measurements do not, therefore, differentiate between the parent drug and its major, closely related, derivatives.

In summary, it can be stated, first, that absorption is critically dependent upon the composition of the oral dosage form with Mygylol being a less satisfactory vehicle than when 3H-OL-27 is dissolved in olive oil. In the former situation, absorption ranges between 2 and 12%, but this may approach 50% in olive oil. It appears that the absorption in humans, when cyclosporin A is given in olive oil, approximates that in dogs. It is of note that the time to achieve peak concentration is also shorter with olive oil, being in the range 2-4 hr, but as long as 24 hr with Mygylol.

Second, the blood levels, which may usefully be compared using the β phase, are seen to have a half-life between 27 hr for humans and approximately 50 hr for monkey, with dog being intermediate. This latter point is of particular interest, since it raises important considerations for scheduling a therapeutic dosage of cyclosporin A.

Third, excretion, when studied after a single dose of cyclosporin A, is influenced by route, formulation, and dose. A fairly consistent pattern is demonstrable between all the animal species studied and humans, with approximately 10% appearing in the urine (range 1.1-23.2%) within the first 96 hr and the remainder demonstrable in feces (range 43.1-90.6%) in the same time period. It therefore appears that renal excretion is only a minor pathway of elimination in all the species tested and is lowest when it is administered in the form of either Mygylol or polyethylene glycol (PEG 200). The major route is via the bile, as indicated by the recovery of radioactivity in the feces. In dog and monkey, excretion is incomplete following an intravenous administration, whereas 80-90% of the radiolabel can be recovered in all species after oral administration. This inconsistency again focuses attention on the differential distribution that cyclosporin A may achieve between compartments and emphasizes the need for further studies on biotransformation.
Fourth, such biotransformation data as are available are most complete for rabbit, dog, and humans, where similar metabolite patterns have been demonstrated in plasma and urine.

Finally, protein binding, particularly in the therapeutic concentration range, shows partition between lipoprotein and red cells, but it is clear that data for the different metabolites are as yet incomplete. This latter consideration may have important bearing on not only assay methods but choice of compartment and cyclosporin level selected to monitor therapy.

C. Dosage and Drug Monitoring

Based on the pharmacokinetic data, no uniform recommendation can be made about cyclosporin A administration in humans. Three generalizations are possible. First, sufficient drug must be given for an adequate period of time to allow equilibration between plasma lipoprotein and other compartments before patients are transferred to maintenance programs. Second, the monitoring of plasma levels by high-pressure liquid chromatography provides different information from that derived by radioimmunoassay. The former measures parent substance only, whereas the latter would include immunologically identifiable analogs, some of which share immunosuppressive properties, albeit less than the parent substance. In this regard, it is not yet known which assay method is preferable, particularly since the relative toxicity of the parent substance and metabolites has not been defined. Thus, the relationship between the administered material and its metabolites with immunological reactivity and toxicity require clarification. Third, the relative value of plasma as opposed to red cell leukocyte concentration has not been fully explored. Initial data using radioimmunoassay show a discrepancy between plasma and red cell levels.

In our studies (66), the red cell levels are consistently higher, so that tissue binding may be underestimated by measurements of plasma level alone. Thus far, we have not been able to clearly define a constant relationship, at any one moment, between levels obtained by radioimmunoassay of serum or red cell hemolysate in patients receiving therapeutic doses of cyclosporin A.

In this state of uncertainty, a sound principle would appear to be the administration of sufficient cyclosporin A to achieve a predetermined plasma level by radioimmunoassay. This dose would then be decreased in the presence of toxic effects or increased with the development of escape phenomena such as rejection or emergence of graft-versus-host disease. Our current practice is to correlate serum levels with those in the peripheral tissue compartment, as reflected in red cell cyclosporin A concentration determined by the same assay.

Four additional points require emphasis. First, in the field of organ transplantation, it has not been established what considerations other than dosage and plasma levels need to be incorporated into the design of immunosuppressive schedules. For example, bearing in mind the time taken to achieve plasma levels following oral administration and the absence of data defining the rate at which tissue compartments are saturated, the period before transplantation in which the recipient should be treated is unknown. Furthermore, at least in the case of allogeneic bone marrow transplantation, the question of pretreating the donor cannot be ignored.

Second, the question of cyclosporin A and drug interaction is still confused. When combined with glucocorticosteroids in vitro, there is experimental evidence to support combination therapy. However, despite studies in a variety of animal species and using
differing experimental models to examine this problem in the allografting of kidney, heart, lung, and bone marrow, there is no uniformity of opinion. Thus, at the present time, the role of cyclosporin A as a single agent or in combination with other drugs or radiotherapy to achieve safe but optimal immunosuppression awaits clarification.

The situation in humans (27) has raised questions about adding prednisolone and a cyclophosphamide derivative to cyclosporin A therapy in patients thought to be rejecting their kidneys. Here, too, the relative merits of this agent alone or in combination continue to be actively debated.

Third, consideration needs to be given to the immunosuppressive properties of cyclosporin A in predisposing patients to severe bacterial and viral infections and, undoubt-
edly, the risk would be increased when combined with other immunosuppressive agents. Similarly, the theoretical possibility exists that cyclosporin A, either singly or in combination with other effective agents, may predispose patients to the emergence of second tumors. Concern, for example, about emergence of lymphomas has already been expressed (68); but the specific situation in the patient with chronic renal disease may be different (69), and there is need for caution to avoid prematurely incriminating this drug in the pathogenesis of second malignancies.

Last, the nephrotoxicity associated with cyclosporin A emphasizes the risk of interaction with other drugs potentially likely to impair renal function. These include the aminoglycoside antibiotics which may be present in plasma concentrations not normally considered dangerous. A similar argument applies to some of the diuretics, and these assume importance in many of the clinical situations where cyclosporin A is given to severely ill patients whose renal function is compromised in consequence of their underlying disease.

Drug interaction with cyclosporin A is clearly important: It is incumbent upon physicians using this agent to be fully informed of the theoretical hazards. On the one hand, there is the need to constantly monitor the patients for the adequacy of cyclosporin levels present in plasma or tissue compartment. On the other hand, one should pay attention to additional complications that may arise in consequence of drug interaction, especially the early biochemical changes of deteriorating renal function.

V. TOXICOLOGY

In experimental models and clinical studies, a number of side effects have been reported. The majority are dose related, being reversible with reduction in cyclosporin A dosage.

A. Experimental Studies

Acute toxicity has been examined in mouse, rat, and rabbit, following oral and intravenous injections. Wide differences were found among species, with 14 day LD50 being, re-
spectively, 107, 254, and greater than 10 mg/kg/day for intravenous route and approxi-
mately 2500, 1500, and greater than 1000 for oral administration. Dyspnea, tachypnea, cramplike movements, stupor, pilo-erection, and death occurred within 3 hr after intravenous injection and up to 9 days with oral administration, diarrhea was a prominent sign with enteral administration.

Chronic studies in rats at oral doses of 14 mg/kg/day showed reduced circulating lymphocytes and a generalized atrophy of lymphoid tissue. At higher doses of 45 and 90 mg/kg/day, death resulted from hepatic and renal toxicity, but in animals surviving after discontinuation of cyclosporin A administration, biochemical tests of hepatic and renal
function returned to normal. There was associated cloudy swelling of renal tubular epithelium and single cell necrosis in the liver. It is noteworthy that the red cell series was reduced.

In dogs, low doses of 5 and 15 mg/kg/day were tolerated without toxicity, but diarrhea and conjunctival hyperemia were encountered at 45 mg/kg/day, food intake was irregularly reduced, and weight decreased. The hematology was altered by an increased erythrocyte sedimentation rate, anemia, and decrease in white cell and platelet counts.

In rabbits, three extensive toxicity studies (66) using New Zealand White males with a starting weight of 3 kg have been completed. The animals were individually housed in stainless steel cages in a temperature-controlled vivarium. Water and complete rabbit diet (Epol, Johannesburg) were allowed freely.

The animals in the first study received a single daily oral administration of cyclosporin A (OL 27-400, Sandoz) in a volume of 3 ml, with a loading dose of 25 mg/kg/day in the first week and 12.5 mg/kg/day in the ensuing 12 weeks. At the end of this period, dosage escalation was undertaken with half the animals being maintained at 25 mg/kg/day and the other half at 50 mg/kg/day for 8 weeks; in a further 8-week period, doses were doubled to 50 and 100 mg/kg/day in the two groups, respectively. In all the animals, blood count, chemistry, and radioimmunoassay for cyclosporin A were undertaken weekly. During this study, all the animals gained weight consistently to an average of 4 kg by day 62. Regular monitoring of plasma cyclosporin A levels showed a peak absorption at 1½ hr following oral dosage and maintenance of plasma levels between 100 and 400 ng/ml that had returned to baseline by 6 hr (Fig. 4). No toxic effects were demonstrable on hematopoiesis, but transient elevations in hepatic enzymes were documented at dosage levels above 50 mg/kg/day. The only consistent abnormality was an increase in plasma creatinine, which promptly reversed on discontinuing therapy.

In a second toxicity study, cyclosporin A was given at doses of 50 and 100 mg/kg/day for 17 weeks. There was a consistent fall in hemoglobin, down to a mean of 10 g/dl, and some minor reduction in platelet count that was not statistically significant. Detailed examination of autopsy material showed generalized bone marrow hyperplasia, focal areas of hepatic necrosis, and cloudy swelling with acute tubular necrosis in the kidneys of some animals.

In the third study, higher doses of 75 mg/kg were given twice a day in a deliberate attempt to define any acute toxic effects that may develop within 6 weeks. In a subsequent 6-week period, doses were doubled, in a stepwise manner, to 300 mg/kg/day on a split schedule. In these animals, the pattern of toxicity was essentially the same as that found at the lower doses. At these higher doses, the absorption peak is delayed and elimination is slower (Fig. 4).

The rabbit studies may be summarized by stating that oral administration of cyclosporin A results in demonstrable serum levels when measured by radioimmunoassay, and even at high dosage, minimal clinical toxicity is found over prolonged periods of study. The only two constant hematologic observations were mild to moderate reduction in hemoglobin level in the presence of a normocellular bone marrow with an intact maturation sequence and variable reduction in blood lymphocyte count. At all dosage levels, reversible biochemical changes of renal dysfunction occurred, reflected by elevations in serum creatinine level, although blood urea usually remained normal. Histological examination of the kidneys showed cloudy swelling and, in occasional animals, acute tubular necrosis. Lymph nodes and spleen had general reduction of lymphoid tissue following prolonged periods of administration, and this was associated with an increased rate of infection late in the course of those animals receiving high doses of cyclosporin A.
Cyclosporin A

Studies on carcinogenesis in a variety of animals have failed to demonstrate embryotoxicity or teratogenicity in nontoxic doses. Similarly, mutagenicity using *Salmonella typhimurium* as an indicator organism was unsuccessful. Tumors did not develop in any of our rabbits.

B. Clinical Trials

1. Patient Tolerance

Both the presently available drinking solution (Sandoz, Basel) and the intravenous preparation are tolerated without any gastrointestinal tract discomfort. With experience now in excess of 3 years, we can endorse the recommendation that the cyclosporin A be given in milk containing a taste-disguising additive such as cocoa, chocolate, or Caotina.

2. Nephrotoxicity

The major side effect of cyclosporin A administration is nephrotoxicity, but many of the studies in humans are difficult to interpret because the drug has been used following kidney grafting or after major surgery for pancreatic or hepatic transplantation (70). A similar problem arises when it is administered either to facilitate engraftment or to control graft-versus-host disease in patients receiving allogeneic bone marrow grafts (28).

In patients undergoing renal grafting, data reporting the effect of long-term cyclosporin A on renal function (71) at a dosage level of 5.9 mg/kg body weight (range 4.0-8.6) were compared with a posttransplantation regimen containing corticosteroids and azathioprine. This study included three renal biopsies which showed nonspecific changes of interstitial nephritis which may reflect low-grade graft rejection or cyclosporin A toxicity. It is noteworthy that plasma cyclosporin A levels are not reported, although the authors indicate that this is desirable. Similarly, cyclosporin A is reported as a feasible immunosuppressive agent in patients with oliguric acute tubular necrosis (72), and this group has initiated a randomized prospective study comparing prednisone with cyclosporin A with prednisone, azathioprine, and antilymphocyte globulin for recipients of either cadaver or live related renal grafts.

Reports on the effects of cyclosporin A in the liver and kidney transplants from America (73) confirm nephrotoxicity between 13 and 22 days of therapy in patients receiving 16.3 ± 2.9 (SEM) mg/kg/day of cyclosporin A, but that kidney function returned to normal with dosage reduction to 9.2 ± 2.3 (SEM) mg/kg/day. Of note was the fact that 4 of 66 patients were switched from cyclosporin A to azathioprine after 4-8 months because of persisting unsatisfactory renal function; in 3 of them graft function improved. The experience of this group is that cyclosporin A is nephrotoxic but that this is easily reversed even after many months of treatment. This study is again limited by the fact that plasma cyclosporin A levels were not used in the decision-making process.

It would appear that the nephrotoxicity is due to a tubular effect (74); although other renal biopsy studies have shown no significant glomerular or tubular lesions (75), and this author has commented on possible protective effects of a mannitol-induced diuresis. Apart from mild and transient elevations of blood urea, nitrogen, and creatinine, glomerular thromboses and severe tubular damage have been reported (76) to correlate with both the clinical course and laboratory findings of severe renal failure in three recipients of allogeneic marrow grafts who were given prophylactic cyclosporin A. These workers emphasized that rising creatinine concentration, accompanied by thrombocytopenia, microangiopathic anemia, and hypertension, suggests cyclosporin A nephrotoxicity.
and that kidney biopsy at the onset of renal failure may help elucidate the early principal lesions, since autopsy data probably represent a combination of effects.

Our studies (66) would suggest that cyclosporin A is toxic to renal tubules in a dose-dependent and reversible manner. In a series of patients receiving cyclosporin A as primary immunosuppressive therapy for severe acute aplastic anemia, without prior allogeneic bone marrow transplantation, a predictable increase in biochemical parameters of renal function correlates with increasing administration of the drug. Creatinine is affected more than the urea level, although both bear a linear relationship to peak and trough cyclosporin A levels measured by radioimmunoassay in serum samples (66). Based on these studies, we endorse the observations of the Swiss group (77) that any increase in serum creatinine levels, particularly while still within the normal range, should be treated by oral administration of loop-acting diuretics. Our practice is to commence 40 mg of furosemide in the morning and, if the biochemical changes have not stabilized or started to reverse within 24 hr, to add a second dose in the evening. Further increase in creatinine level on this regimen is managed by 25% reduction in cyclosporin A dosage while monitoring plasma levels. Used in this way, we have found nephrotoxicity related to cyclosporin A an easily controllable problem. Red cell RIA is now recommended as preferable to plasma assay.

These observations have been extended to patients following allogeneic bone marrow transplantation and, to date, such meticulous control of dosage with regular biochemical monitoring has resulted in equal ease of control of cyclosporin A toxic effects.

Two points require additional comment. The administration of cyclosporin A with other potentially nephrotoxic drugs, notably, the aminoglycoside antibiotics, results in deteriorating renal function despite antibiotic levels being monitored and remaining in the supposedly nontoxic range (78). Our own practice has therefore been to avoid any agent with the potential for interfering with renal function. Currently, antibiotics of choice are third-generation cephalosporins, and their use has virtually abolished our previous experience of renal failure related to drug administration.

Second, with regard to histological changes, those evident on light microscopy (76) are supplemented by ultrastructural changes (79) which suggest that cyclosporin A, or its metabolites, may interfere with mitochondrial metabolism.

3. Hepatoxicity

Biochemical evidence of liver dysfunction, in which the hyperbilirubinemia may progress to jaundice, has been reported in association with cyclosporin A administration (80,81). It is, however, necessary to bear in mind that in many of these patients, notably those following allogeneic bone marrow transplantation, other factors such as graft-versus-host disease (GVHD), septicemia, and drugs known to be hepatotoxic may, independently of cyclosporin A administration, produce similar changes. The way in which cyclosporin A compounds the problem remains unresolved. It has been suggested (80) that hyperbilirubinemia is a toxic consequence of cyclosporin A therapy rather than the cause, and it has been postulated (78) that because cyclosporin A is excreted in the bile there may be competition with the pathway used for bilirubin clearance. If this is correct, then any factor inhibiting the excretory pathway of the latter may impair excretion of cyclosporin A, leading to increased plasma levels.

Our experiences with patients undergoing allogeneic bone marrow transplantation and with those receiving cyclosporin A as a primary form of treatment for severe acute aplastic anemia, where no bone marrow transplantation option exists, show a clear
temporal relationship between rising cyclosporin A levels and the development of hyperbilirubinemia, as reported by others (81). It appears that cyclosporin A-induced hyperbilirubinemia occurs with trough levels that are consistently above 400 ng/ml; accordingly, there is a need to monitor plasma levels three times a week. We have found that reduction of cyclosporin A levels will lead to a decrease in bilirubin level, provided this is not raised for some other reason, of which we have found sepsis to be the most common.

To further define the possible mechanism, an isolated rat liver model has been used (82). In this in vitro study, a variety of biochemical techniques have consistently failed to demonstrate cyclosporin A in association with ligandin or the Y-transport protein in hepatic cytosol. This evidence would suggest that competition between bilirubin and cyclosporin A for hepatic proteins is not the mechanism by which their efflux is regulated. The finding of cyclosporin A in association with the 15,000-dalton fraction is compatible with hepatic excretion via the minor or Z-protein-mediated pathway. However, a number of other enzymes, peptides, and low molecular weight proteins found in the same area have made it impossible to definitively incriminate cyclosporin A competitive binding even in this quantitatively less important route for bilirubin excretion.

It therefore appears that hepatotoxicity, and particularly hyperbilirubinemia, is a consequence of a rising plasma cyclosporin A level which, in our experience, invariably follows biochemical evidence for impaired renal function. The mechanism by which cyclosporin A exerts its hepatotoxicity is presently unknown, but direct competition with the bilirubin transport system along the ligandin pathway in the liver seems unlikely.

4. Hypertension

In patients receiving cyclosporin A, systemic hypertension and convulsions have been reported (83) and, while the pathogenesis is not clear, the available data suggest that impaired renal function causes fluid retention which promotes these events. The complications seem directly related to cyclosporin A blood levels, with younger patients being more susceptible to the toxic effects of this drug.

5. Infection

In the immunosuppressed patient following cyclosporin A administration, herpes simplex, herpes zoster, and cytomegalovirus infections have been reported (70). In the Cape Town clinical experience, the incidence of viral or bacterial infections in patients receiving cyclosporin A for aplastic anemia, who have not undergone allogeneic bone marrow transplantation, is no different from the control population. Furthermore, this finding holds true for patients undergoing allogeneic bone marrow transplantation whether they received methotrexate immunosuppression or only cyclosporin A.

6. Immunosuppression and Neoplasms

Breast lumps have been reported in two patients (84). The question of malignant disease in patients receiving cyclosporin A is complicated by the immunosuppressive effects that may be associated with end-stage renal disease and cell-mediated immunity in these patients (85). Nevertheless, concern about lymphoma developing in patients on cyclosporin A started with the report of the Cambridge group (70). Subsequently, this risk has been reemphasized (54,68,86) and, since there is a sound immunological basis for considering this possibility, caution is necessary (69).
7. Other Toxicities

We have not encountered gastrointestinal tract intolerance, pancytopenia, lymphocytopenia, or gingival hypertrophy. Hirsutism has been seen in 1 of 50 patients, and extrapyramidal signs of neurotoxicity elicited by careful examination in 5 of 50 patients, but this was short-lived in 4 and required drug dosage reduction in only 1.

VI. EXPERIMENTAL MODELS

A. Kidney Allografts

A number of experimental animal systems have established, in broad terms, the effectiveness of cyclosporin A administration in maintaining graft function and survival despite histoincompatibility. Apart from species differences, other important variables are dosage, scheduling, and mechanisms by which tolerance is induced.

In rats (87-89), cyclosporin A conferred a clear dose-related advantage to survival of kidneys transplanted from DA to Lewis strains, despite the existence of a strong histocompatibility barrier. Histological studies showed the presence of mononuclear cell infiltration, although function remained excellent. This evidence would suggest that the lymphocytotoxin or humoral antibody-mediated response, leading to acute rejection, was suppressed, whereas the cell-mediated cytotoxicity, reflected in the mononuclear cell infiltration, was little altered.

A second consideration is the presently unexplained way in which this immunosuppressive advantage is lost at high dose. Thus, although plasma levels are not quoted, when cyclosporin A was administered intravenously at 5 mg/kg/day for 14 days (90) or orally (91), levels high enough to impair renal or hepatic function lead to a paradoxical loss of immunosuppression, but it is not clear whether this represents an effect on the donor kidney or an alteration in the host immune response. These observations were extended by studying renal allograft survival in which the recipients had been previously sensitized by skin grafting for up to 5 weeks prior to kidney transplantation (88). The studies showed the drug to be relatively ineffective in suppressing rejection of a renal allograft in a previously sensitized recipient. Of particular importance is the scheduling of the immunosuppressive agent in relation to the transplantation procedure. Thus, neither pretreatment alone nor commencement after a delay of 4 days following transplantation appears effective in suppression of rejection (89,92).

In rabbits, confirmatory data have been reported. At 25 mg/kg/day by mouth, 18- or 28-day courses prolong survival of renal allografts (56,93), while untreated animals died from renal failure associated with acute rejection. In an autografted group from the same study, the survival was not statistically different from those receiving cyclosporin A. Of note was the fact that 6-mercaptopurine was less effective than cyclosporin A in maintaining graft survival. In addition, the latter agent had the unusual property of its immunosuppressive effect persisting in the rabbits after therapy had been discontinued, and this was equivalent whether given by the intramuscular or oral route.

To define the specificity of this tolerance (94-96) experiments using both skin grafts and second kidney transplants from the original or third-party donors were carried out. The accumulated results of the studies were not uniform. One group (94) interpreted the data as reflecting donor but not tissue specificity while another (96) considered the tolerance to be nonspecific. It was pointed out (97) that the discrepancies may in part be explained by the delayed appearance of donor-specific tolerance which is known to occur in the rat (57,98) and in part by the different assay methods used.
In the rabbit, mononuclear cell infiltrates were found in kidney biopsies; but their interpretation remains controversial, and Green (99) raised the possibility that cyclosporin A may have attenuated mainly the humoral antibody response or alternatively could have inhibited migration of lymphocytes. Furthermore, the author questioned whether the cells were T helper in nature or whether the phenomenon was a concentration effect because there were insufficient lymphocytes to mount an aggressive attack and destroy the organs. The latter would be a manifestation of the quantum theory (100) so that the kidneys simply underwent slow attrition over a period of many months.

In dogs (101), early studies demonstrated the immunosuppressive properties of cyclosporin A and showed these were superior to azathioprine but were less spectacular than in rats and rabbits, despite which, some animals survived for long periods after cessation of all therapy (99). In subsequent systematic studies (102), the effects were confirmed, and rejection was completely suppressed with a clear dose-response relationship (103). Of note was the fact that rejection occurred approximately 2 weeks after withdrawal of the cyclosporin A, and this response differed from that observed in the rat. Another point of distinction between species was the observation that cyclosporin A combined with prednisolone did not result in the adverse effects in dogs that had been noted in rabbits (104) and, furthermore, conversion of the immunosuppressive regimen from cyclosporin A to conventional drugs such as azathioprine or prednisone was possible (105,106).

Perhaps surprisingly, reversal of acute rejection could also result from cyclosporin A administration 4 days after allografting (107) which differs from the analogous experiment in rats and rabbits (99).

B. Allogeneic Bone Marrow Transplantation and Graft-Versus-Host Disease

In mice, Borel et al. (17) demonstrated a capacity for cyclosporin A to allow reconstitution of hematopoietic and lymphoid tissues by incompatible spleen cells and to delay the emergence of lethal graft-versus-host-disease.

In rats (45), cyclosporin A provides adequate immunosuppression both for graft acceptance and prevention of subsequent graft-versus-host disease. The animals develop a chimerism with normal immune response and repopulation of T-cell areas in lymphoid organs. The establishment of a chimeric state with specific tolerance to donor and recipient but not to third-party grafts may reflect the generation of a suppressive cell population (108,109).

In the rabbit, conflicting results have been obtained. Histoincompatible skin grafts were possible in a model where red Burgundy donor and outbred New Zealand White recipient animals were used but, at doses of 15 mg/kg subcutaneously, while engraftment occurred, graft-versus-host disease could not be prevented. The experience in Cape Town (110) is different. Using incompatible NZW and R strain animals, there is a statistically significant difference in animals receiving 10 mg/kg/day of cyclosporin A by intramuscular injection for 28 days after transplantation. Thus, at 40 days only 10% of allograft control animals were alive, whereas 44% of those receiving cyclosporin A survived. This difference was even more obvious at 100 days when none of the control animals survived but a third of those that were treated were still alive and well, with normally functioning donor grafts. Furthermore, the histologically recognizable features of graft-versus-host disease were present in all the control animals and in only 25% of those receiving cyclosporin A. It appears that in this animal model, species differences may be critical in trying to understand the differences between results obtained by the Swiss and the South African
groups since, in most other respects, the experimental animal models were remarkably similar.

In dogs (41, 111-113) cyclosporin A increased the incidence of graft failure, although with engraftment median survival is prolonged and the onset of graft-versus-host disease is delayed, despite the failure to induce stable tolerance.

C. Skin Allografting

In mice (29), cyclosporin A was comparable with antilymphocyte serum for graft retention although in subsequent studies (114,115) the immunosuppressive action was demonstrable only as long as cyclosporin A was biologically active and rejection took place between 10 and 14 days after discontinuing the treatment. A similar dose-dependent prolongation of skin graft survival has been reported by other workers (116).

In rats (117), essentially the same phenomenon occurs but may be modified in animals receiving a heart graft. From this, it was suggested that when a large mass of foreign tissue is tolerated, this may be sufficient to block rejection of skin graft, whereas the smaller volume involved in transfer of skin only is incapable of exerting this effect (99).

D. Cardiac Allografts

In rats, heterotopic cardiac transplantation (118) established the potency of cyclosporin A with even short courses, preventing rejection for prolonged periods of time. Subsequently (23), attention was focused on this phenomenon, but these studies are difficult to interpret because information about plasma cyclosporin A concentration, or that found in other cellular compartments, is not available.

In rats, xenografting (119) from outbred Syrian hamsters to the renal vessels of Lewis rats results in modest prolongation of the donor heart while on an oral course of cyclosporin A of 35 mg/kg/day.

In nonhuman primate heterotopic grafting (120), it was established that cyclosporin A is superior to azathioprine and corticosteroid immunosuppression, but that prolonged treatment with this antifungal agent singly or in combination with other drugs may be necessary to maintain function.

In pigs, orthotopic grafting (24) is of particular interest since survival of control animals mismatched at the major locus was 6 days, whereas those animals to which cyclosporin A was administered at a dose of 24 mg/kg/day by intramuscular injection followed by oral maintenance had mean survival times of 265 days. The animals were healthy, without biochemical evidence of hepatic, renal, or hematologic abnormality, and established not only the effectiveness of immune suppression for cyclosporin A in this model but the fact that survival was possible for prolonged periods of time without additional treatment.

Nonhuman primate orthotopic cardiac allografting, which has important parallels for clinical practice, has been extensively studied (120-123). These experiments show that function is prolonged even in association with mononuclear cell infiltration and myocyte injury. As in the heterotopic grafting, cyclosporin A exerts a powerful immunosuppressive effect which is superior to other currently available agents; however, prolonged administration is necessary, and it may well be required in combination with other agents for optimal preservation of function.
E. Pancreatic Allografting

In rats, with segmental and pancreatic islet transplants (124-126) it has been demonstrated that cyclosporin A has a relatively modest effect, although another study (127) has been more successful in inducing long-term survival in the diabetic animals. An attempt to define reasons for both the differences found in these studies and also between results obtained with pancreas and other tissues has been made by exploring a possible role for the macrophage. Indefinite graft survival followed injection of 50 mg/100 g body weight of silica (128), suggesting that this cell system may play a special role in transplantation of this organ.

In dogs, allografting of the pancreas has been undertaken using vascularized segmental grafts, and prolonged survival is possible at high doses of cyclosporin A (129). While neither prednisolone nor azathioprine prolonged survival of animals compared with untreated grafted controls, cyclosporin A at a dose of 25 mg/kg/day orally extended the period of normoglycemia from a mean of 9 to 85 days, and of individual survival from a mean of 13 to a maximum of 129 with a mean of 72 days.

F. Lung Allografting

In dogs (130), it has been shown that survival of this organ for over 5 months was possible with cyclosporin A administered in oral doses of 17 mg/kg/day for 35 days, after which the drug was gradually reduced and given concurrently with low-dose azathioprine (2 mg/kg/day for 14 days). It was remarkable that rejection occurring while on this regimen was later in onset and more easily reversed by high-dose corticosteroids than that developing on standard immunosuppression.

Nonhuman primates (123) have a long survival following lung transplantation with cyclosporin A therapy and, apart from mild lymphocytic infiltration, rejection injury is minimal. This is particularly attractive because bacterial infection is reduced and healing mechanisms appear better preserved. It is currently necessary to define dosage and schedule, to establish whether simultaneous administration of other drugs including corticosteroids may confer additional advantages and, since malignant lymphoma may occur in the primates under these circumstances, to investigate the mechanism of tumor induction.

G. Corneal Allografting

In the rabbit (131,132), skin grafts were placed from the same donor 14 days later to ensure rejection of the cornea. In control animals, the corneal grafts were rejected promptly in contrast to a 50% survival when animals received cyclosporin A at a dosage level of 25 mg/kg by intramuscular route for 28 days from the time of the operation. With a shorter period of administration (14 days), the survival period was intermediate between control and 28-day administrations. Skin graft survival was also prolonged but invariably shorter than the cornea.

Topical application of cyclosporin A was not effective in preventing graft rejection, and it is speculated that this may be due to the scheduling of cyclosporin A and the provocative antigen. It also remains uncertain whether the physical and biological stability of the immunosuppressive agent is altered in the aqueous medium on the surface of the eye.
H. Skeletal Muscle

Cyclosporin A has been demonstrated to permit persistence of an allograft in A and CVA mice, whereas in the nontolerant hosts the allografts were rejected between the tenth and the twelfth day (133).

I. Nerve and Schwann Cells

It has been demonstrated (134) that both minor and major antigens evoke an immune response that prevents functional regeneration in nerve allografts. Nerve fibers, however, regenerate in immunosuppressed hosts. In an extension of these studies (135), it was demonstrated that cyclosporin A can prevent the rejection of nerve allografts with host axon regeneration through long nerve segments.

J. In Vivo Cell-Mediated Immune Reactions

The delayed hypersensitivity skin reaction to oxazolone in mice (20) defined by the suppressive index induced in the skin reaction was examined for both primary and secondary response. It emerged that when cyclosporin A, cyclophosphamide, and azathioprine were compared, the most favorable ratio between toxic and pharmacological effects was found with cyclosporin A.

In guinea pigs, the delayed skin reaction to tubercul in (21) showed a clear advantage for cyclosporin A over large doses of phenylbutazone and azathioprine. The data also supported differences in action between cyclosporin A and the anti-inflammatory agent, phenylbutazone, with the former inhibiting effector cells at clinically well-tolerated doses, whereas phenylbutazone produced severe side effects; azathioprine, although impairing the hypersensitivity reaction, was associated with deaths in some of the animals.

Cell-mediated cytolysis in mice (16) was characterized by clear dose-dependent inhibition of allogeneic target cell lysis by specifically sensitized lymphoid cells. In this assay system, cyclosporin A was compared with procarbazine, cyclophosphamide, the epipodophyllotoxin VP 16-213, and antilymphocyte serum. The results showed a general cytostatic action of procarbazine, cyclophosphamide, and the epipodophyllotoxin whereas cyclosporin A, in common with antilymphocyte serum, appeared primarily to affect lymphocytes at the stage of sensitization.

K. Immune Experimental Models

1. Experimental Allergic Encephalomyelitis (EAE)

In rats, primary sensitization with an emulsion containing spinal cord and killed dried Mycobacterium smegma caused the development of paralysis in control animals (39), and some 8-10 days later in those treated with cyclosporin A at the time of sensitization (39,136). A greater effect was obtained with oral doses of cyclosporin A between 50 and 100 mg/kg than was seen when azathioprine was given as a reference drug. It appears that in this particular test model, cyclosporin A inhibits sensitization and suppresses the effector phase but is not curative, since withdrawal of treatment is followed by development of symptoms after a lag period of 1-2 weeks.

In guinea pigs (137), cyclosporin A was highly effective in suppressing EAE at doses of 35 mg/kg, given on either a prophylactic or a therapeutic schedule. Of note was an unexplained sex difference in the response to drug, with the greater effect being evident in females. Predictably, prophylaxis was the more effective and such therapeutic benefit
as occurred may reflect prevention of T-lymphocyte recruitment to perpetuate the inflammatory reaction.

In the rhesus monkey (138), paralysis killed control animals between day 22 and day 42. In the test group which received 50 mg/kg of cyclosporin A every alternate day, the disease was suppressed, and one animal relapsed but responded to intramuscular injections of the same dose. A similar finding was present in a second animal, while two others developed no neurological signs. Secondary challenge 204 days after beginning the experiment did not alter progression of the established disease. Of note is the finding that two of these animals never developed the disease while on cyclosporin A, despite sensitization. The ability to mount a secondary response is seen as evidence that priming must have taken place, leading to the formation of memory cells.

The information obtained in these studies on experimental allergic encephalomyelitis is of relevance for similar demyelinating diseases in man, such as multiple sclerosis and postinfectious encephalomyelitis.

2. Freund Adjuvant Arthritis

In rats (21), when comparing the effects of cyclosporin A, phenylbutazone, and azathioprine on the prevention and treatment of arthritis, the most striking benefits were found when 30 mg/kg/day of cyclosporin A was given orally on a prophylactic schedule with some limited inhibition also occurring once the arthritis had become clinically manifest. On the preventive schedules, cyclosporin A appeared better than both phenylbutazone and azathioprine, but the expression of the ED₅₀ on a milligram per kilogram basis may not be the most valid basis for comparison. Once arthritis was established, the drugs were given from days 14 to 20 inclusive, and here, azathioprine was not effective, while there was little to choose from between phenylbutazone and cyclosporin A. It was pointed out (21) that the peptide is inactive in the acute inflammatory process, and is without effect in carrageenan paw edema and other such models.

3. Experimental Autoimmune Uveitis

In rats (139), cyclosporin A was effective when given concomitantly with the retinal S-antigen at 10 mg/kg, and caused partial prevention of uveitis down to 5 mg/kg/day. Even when the daily injections were started 7 days after immunization, treatment was effective, but here a higher dose of 40 mg/kg/day was needed to suppress the inflammatory process in the eye.

4. Lupus Erythematosus

In New Zealand mice, the classic model for the study of this autoimmune entity, proteinuria and mortality were the end points of a study (136) comparing the use of cyclosporin A with cyclophosphamide, and showed that at 20 mg/kg/day, cyclosporin A had no effect on proteinuria. This physical sign was, however, markedly suppressed when doses were increased to 60 mg/kg/day, and the result was the same as could be achieved with cyclophosphamide at 60 mg/kg/day given in a 10-day course. Mortality increased gradually through the 18 months of the study, with the first deaths occurring at 8 months and reaching 50% at 12 months. Cyclosporin A was less effective than cyclophosphamide in protecting animals from the autoimmune disease in this model, and therefore the interpretation and extrapolation of relatively incomplete data in humans would be inappropriate. Further detailed characterization is necessary, including patterns of response in other
Experimental animals. While potentially of value, the preliminary data suggest the need for caution in using cyclosporin A in patients with systemic lupus erythematosus.

5. Experimental Infections

Neither bacterial infections in mice nor fungal infestations in mice or guinea pigs appear to be adversely affected by cyclosporin A administration. Viral infections in mice and guinea pigs are marginally altered by cyclosporin A, but currently the evidence is insufficient to incriminate this substance in aggravating this risk.

An antimalarial effect has been reported for cyclosporin A in the mouse (140) in which cyclosporin A started concurrently with the inoculation of the *Plasmodium berghei* or *chabaudi* were less effective than treatment started when parasitemia was already established. The available evidence suggests that the antimalarial action results from direct toxic effect to the parasites.

In the mouse (141), cyclosporin A has been reported to exert an effect on *Schistosoma mansoni* by significantly reducing the number of mature and immature male, and to a greater extent female, worms.

VII. CLINICAL TRIALS

A. Renal Transplantation

The initial study with cyclosporin A in man (27) was in seven patients on dialysis with renal failure who were given kidneys from mismatched cadaver donors and immunosuppressed with intramuscular cyclosporin A for 3 postoperative days and then maintained on oral administration. While establishing clearly the value of cyclosporin A in clinical renal transplantation and the fact that this occurred without marrow toxicity, two other important points emerged. First, while there was no histological evidence for severe rejection, a number of patients had poorer function immediately after transplantation than had been anticipated, necessitating the addition of the other immunosuppressive agents, prednisolone and cytisine, on the basis that low-grade rejection was occurring. In these patients, nephrotoxicity, reflected in raised serum creatinine and urea levels, improved when cyclosporin A dosage was reduced or the drug was withdrawn.

The Cambridge experience was then extended (70), confirming the value of cyclosporin A in the immunosuppression of cadaveric organ grafts and drawing attention to the higher incidence of infection, often lethal, when cyclosporin A was combined with prednisolone or alkylating agents. The occurrence of lymphoreticular malignancy was noted in these patients (142). In addition, when this drug was used in patients who were well hydrated (143), 1-year graft survival was 86%; 11 of 32 patients retained good function for between 3 months and 2 years, never having received steroids; and 13 others required these agents for the treatment of rejection only. The nephrotoxicity associated with cyclosporin A can be anticipated from regular monitoring of plasma or whole blood levels with appropriate dosage adjustment if necessary.

In a controlled trial (97), cyclosporin A was compared with the established azathioprine, low-dose prednisolone regimen in diuresing patients who have received HLA-DR incompatible kidneys. With 35 patients studied, graft survival was comparable and, furthermore, this improved on conversion to azathioprine and prednisolone. The presence of cellular infiltrate in biopsy specimens has led these workers to consider steroids
necessary in conjunction with cyclosporin A with the intention of avoiding longstanding renal damage from rejection.

At least two other clinical experiences can now be analyzed, since sufficient numbers of patients are available for study. In the United States (144,145), cyclosporin A has been combined with prednisolone, and again there have been graft survivals of approximately 80% at 1 year. These investigators consider it important to use corticosteroids in conjunction with cyclosporin A rather than the latter agent on its own.

On balance, there is little doubt that the experience in experimental animals applies also to allografting in humans. There is a consensus that cyclosporin A is effective, but the major unresolved issue is whether this should be used as a single drug or whether long-term benefit will accrue from combination with other agents, such as prednisolone, and perhaps in combination with azathioprine. To a lesser extent, but nevertheless of major importance, are the side effects. These include gum hypertrophy, hirsutism, tremor, and hepatotoxicity with the problems of infection and the possibility of lymphoma in the immunocompromised host still to be defined.

B. Bone Marrow Transplantation

In the initial study (28), cyclosporin A was given to five patients with acute leukemia for the treatment of graft-versus-host disease, with poor results. In an extended study (146), cyclosporin A was used in combination with allogeneic sibling bone marrow transplantation to treat 23 patients, and 15 are reported to be in good health; 1 has developed florid graft-versus-host disease.

In Cape Town, the initial experience in a small number of patients treated with cyclosporin A alone for immunosuppression following allogeneic bone marrow transplantation showed no difference in the incidence of acute or the later development of chronic graft-versus-host disease. The one remarkable observation, however, was that where patients on cyclosporin A did develop acute graft-versus-host disease, this could be rapidly controlled with high-dose prednisolone, whereas we have previously been unsuccessful in managing fulminating GVHD with any currently available immunosuppressive agent.

On the basis of our experience, two considerations have arisen. The first of these is that time- and dose-response curves show a lag period of some 7 days before adequate trough levels are found in the plasma after oral administration of 25 mg/kg/day, suggesting that some time is necessary to saturate peripheral compartments. Under these circumstances, the rationale for giving cyclosporin A together with the donor marrow graft is suspect, and recipients therefore receive cyclosporin A long enough before the antigen to stabilize serum trough levels between 200 and 350 ng/ml and peak levels between 500 and 750 ng/ml.

A second and equally important consideration is whether the donor should be pretreated with cyclosporin A. On the basis that graft-versus-host disease, particularly the acute variant, may reflect transfer of immunologically competent lymphocytes to the recipient, we have followed this policy in the last six patients. All donors take cyclosporin A only after having been fully apprised of the risks and after having given informed consent. To date, no donor toxicity has been encountered, and it is remarkable that the last six consecutive transplant patients have been devoid of graft-versus-host disease. These initial observations require confirmation.

Experience with one patient was initially perplexing in that she developed GVHD on the regimen of donor and patient pretreatment with cyclosporin A where we would
not have anticipated this. Plasma levels showed immeasurable quantities of cyclosporin A, and the patient was insistent that she was taking the drug. However, on admission to hospital, measurement of the dose-response curve showed a prompt oral absorption. Unfortunately, grade 1 cutaneous GVHD had emerged in a butterfly distribution on her face, and while this has not extended, it has also not resolved with continued cyclosporin A and prednisolone treatment. This finding is in keeping with our experience that reversal of established GVHD is less satisfactory than prophylaxis with cyclosporin A.

Two points are of particular interest. The first of these is the question of nephrotoxicity which we find to occur universally in our patients. We completely endorse the Swiss experience (147) that meticulous regular monitoring of plasma urea, creatinine, and clearance levels is mandatory. Any rise in the creatinine level, even while in the normal range, is managed by low-dose loop-acting diuretic and, under these circumstances, control is rapidly achieved providing peak and trough plasma levels are in the arbitrarily defined therapeutic range. Rarely is it necessary to reduce the cyclosporin A dosage in patients because of deteriorating renal function when early management to promote diuresis has been started. In one patient, failure to appreciate the gravity of a rising creatinine level required drug withdrawal and, on continuing diuresis, renal function returned to normal within 14 days. Creatinine clearance, corrected for surface area and renal biopsy, was normal, and the patient subsequently tolerated further cyclosporin A therapy without undue sensitivity to the drug.

We consider the concomitant use of nephrotoxic agents, notably aminoglycoside antibiotics, particularly hazardous. It has therefore been our policy to replace these with third-generation cephalosporins which are considerably less damaging to the kidney, and we are convinced that the drug interaction, leading to impaired renal function on the basis of cyclosporin A administration, has sharply decreased.

The second point is the development of hepatotoxicity, and we have yet to see our first patient where this has occurred ahead of renal failure. When biochemical disturbances in liver function arise, the release of hepatic enzymes into the plasma usually occurs and, in our opinion, reflects GVHD. Hyperbilirubinemia occurs in occasional patients where cyclosporin A administration is improperly controlled or where adequate management of deteriorating renal function has not taken place. Two patients managed with less frequent clinic visits than we consider optimum, because of domestic circumstances, have had transient periods of jaundice. In both, renal function has sharply deteriorated, cyclosporin A levels were high, and in both, drug discontinuation, forced diuresis, and correction of plasma levels of cyclosporin A resulted in bilirubin returning to normal within 21 days. These patients remain on cyclosporin A without the development of GVHD and without further complications, but require close clinical supervision.

On the basis of our pilot study, we believe that pretreatment of donor with cyclosporin A to a standardized plasma or preferably whole blood level can be achieved with minimal side effects and may beneficially influence incidence and severity of acute GVHD. To define this schedule further it is presently being randomly and prospectively compared to a control group in which donors are not pretreated; the recipients in both arms of the trial start CSA 14 days before autograft and continue for at least 180 days.

C. Liver Transplantation

Clinical experience (70,143,144) has been encouraging and doses of 10 mg/kg have not been associated with major hepatotoxicity. However, rejection remains a problem, and it has been concluded (144) that cyclosporin A must be combined with prednisone.
D. Pancreas Transplantation

To date, clinical experience has been limited (70), but the attraction of an immunosuppressive agent which would make it unnecessary to use corticosteroids with their profound effects on carbohydrate metabolism cannot be denied. The published data are insufficient to be able to see the procedure of vascularized segmental pancreatic allografting in any role other than developmental, but the preliminary experience with cyclosporin A (99) in immunosuppressive therapy is encouraging.

E. Nontransplantation Experience

1. Rheumatoid Arthritis

In this condition (148), data are insufficient for any critical evaluation. However, it is interesting that gastrointestinal tract intolerance with reversible nephrotoxicity occurs and that some degree of hepatotoxicity is reported. It is difficult, however, to establish the time sequence between toxic effects on the kidney and on the liver in this study.

2. Severe Acute Aplastic Anemia

In Cape Town, a prospective randomized clinical evaluation has been carried out in patients who meet the internationally defined criteria for severe acute aplastic anemia, but who lack a donor option, to compare cyclosporin A with cyclosporin A in combination with antilymphocyte globulin. The patients were evaluated immunologically with cell membrane surface marker studies and for in vitro erythroid colony growth in the presence of the immunosuppressive agent. By such parameters, no response to cyclosporin A has been obtained. Of particular note has been the opportunity to study, over a mean period of 2 years (range 6 months to 3 years), cyclosporin A toxicity in these patients whose course is not complicated by the severe morbidity of allogeneic bone marrow transplantation.

Quite clearly, nephrotoxicity occurs on a reversible basis. Early recognition of a rising plasma creatinine, while peak and trough plasma cyclosporin A levels are still in an established therapeutic range, can often avert progression by the addition of small doses of loop-acting diuretics. In two patients who followed this course and then underwent renal biopsy, only nonspecific changes were found. Hyperbilirubinemia was encountered in three patients where very high cyclosporin A levels were present in the plasma and where renal function was severely compromised. In each of these, renal function returned to normal within 28 days following the start of diuretics and adjustment of cyclosporin A plasma levels, and bilirubin level returned to normal more slowly but did not remain elevated in any.

In the second phase of the same clinical trial, patients failing to improve on cyclosporin A, with or without antilymphocyte serum, are receiving a 6-month trial of additional anabolic androgen and corticosteroid therapy. None of these patients has improved, despite the longer period of treatment, and no additional toxicity has been noted. Perhaps surprisingly, hepatotoxicity associated with anabolic androgen administration has not been a problem.

In the second phase of the same clinical trial, patients failing to improve on cyclosporin A, with or without antilymphocyte serum, are receiving a 6-month trial of additional anabolic androgen and corticosteroid therapy. None of these patients has improved, despite the longer period of treatment, and no additional toxicity has been noted. Perhaps surprisingly, hepatotoxicity associated with anabolic androgen administration has not

3. Psoriasis

In psoriasis (149), there has been dramatic reduction reported in skin lesions, but these recur on discontinuing therapy.
4. Primary Biliary Cirrhosis

In primary biliary cirrhosis, a significant decrease in the ratio of helper to suppressor T cells has been reported (150); these changes are not seen in other forms of cholestatic liver disease but bear a close similarity to those described in chronic GVHD. In this study, the proportion of suppressor T lymphocytes and liver function improved in six patients, confirming the immunoregulatory action of cyclosporin A, but prolonged studies were precluded by nephrotoxicity.

VIII. COMMENTS

The scientific publications on the pharmacology, immunology, experimental studies in animals, and clinical trials of cyclosporin A increase daily. Despite all this work, a considerable time will need to elapse before the role that cyclosporin A comes to occupy among the immunosuppressive agents is defined. Nevertheless, the availability of this unique fungal metabolite represents an advance in current immunosuppressive therapy. In order to realize the potential that this agent has, both in basic immunology and in clinical practice, effort will be needed to resolve a number of important issues.

First, the specific action of cyclosporin A remains controversial. The bulk of evidence favors interference with a particular molecular event involving interleukin 1 early in antigen recognition. This phenomenon finds its expression as impairment of those immunological responses that depend upon T-lymphocyte help for their optimum activity. Not surprisingly, both humoral and cell-mediated mechanisms have been reported as being defective in the presence of this agent. Of fundamental scientific interest is the need to disentangle the effect on lymphocyte subpopulations and their interactions with other cells of which the monocyte-macrophage system is of special interest.

Second, there is the question of pharmacology. While preliminary observations are available for experimental animals and for humans, it is still not known how best to monitor cyclosporin A therapy. Should plasma or whole blood measurements be used to anticipate the development of toxicity? What is the best method to recognize the maximal immunosuppressive effect that can be achieved? Superficially, these two considerations would appear to be related. However, there is currently no secure basis for the belief that a given plasma or whole blood level will directly correlate with optimum immune suppression of immune integrity. Thus, the current practice of determining plasma levels only provides evidence that the drug is being taken. Most investigators have established broad guidelines to integrate these observations with careful consideration of biochemical changes in renal and hepatic function to anticipate the development of toxic but reversible side effects. It remains to reach agreement on the relative merits of radioimmunoassay as opposed to high-performance liquid chromatography, the compartment to be sampled, and the therapeutic index for cyclosporin A.

Third, much of the toxicology requires confirmation. Although reversible nephrotoxicity is recognized, the correlation of functional with morphological changes, particularly at the ultrastructural level, is in its infancy. Serial studies in experimental animals and clinical studies are needed to define late sequelae and test proposals for drug scheduling where other nephrotoxic agents are unavoidable. A similar degree of uncertainty characterizes liver damage. It is not clear how changes in bilirubin level or enzymes are brought about. In marrow transplantation, there is the added problem of graft-versus-host disease disturbing hepatic biochemical function and how these two are to be separated with certainty. The temporal sequences relating disturbances in renal and hepatic function are
not clear and, until the mechanisms are established, decisions made for patient management must remain largely anecdotal.

Fourth, the many experimental models used and the enormous variation in evaluating cyclosporin A have added to the confusion. Despite meticulous examination of the original experimental reports, painstaking reanalysis of data, graphs, and tables, and attempts to match results from different studies, many insoluble questions remain unanswered. For example, the formulation of the drug has changed during the course of these investigations so that route, schedule, and presentation are not uniform. Few, if any, of the studies have reported measurements of plasma levels, and no data are available on tissue concentrations. Despite these shortcomings, there is now a large block of information which supports an unequivocal immunosuppressive activity for cyclosporin A. Future studies will benefit from the availability of standardized assay techniques, and the experimental model remains a major source of information in urgently answering some of the questions. These would include, for example, the type of infection or the emergence of tumors that may occur in individuals immunosuppressed with cyclosporin A. Another question would be the schedule for allogeneic bone marrow transplantation and our suggestion that donor and recipient pretreatment may be the critical issue in abrogating graft-versus-host disease rather than restricting the agent to the recipient. The answer to these and other questions will in large part be derived from properly designed and meticulously executed studies, but attention must now be given to the inclusion of immunological and pharmacological monitoring in data reporting.

Fifth, the clinical trials are in many areas confirming encouraging results obtained from animal studies. Experience is limited, and many of the same reservations apply to humans as to experimental animals. Undoubtedly, clinical trials with cyclosporin A are now a reality, but results are not uniform. Again, interpretation is bedeviled by changes that have occurred with improvements in drug formulation, often the failure to correlate results with standardized plasma levels, and paucity of data on concurrent monitoring of changes in humoral and cell-mediated immunity. There would be little excuse for persistence with trials that failed to take into account such fundamental requirements. On this basis, a period of consolidation lies ahead. An issue that requires urgent attention is the value of cyclosporin A as a single agent or its role in combination with other immunosuppressive drugs, ranging from antithymocyte globulin through adrenocorticosteroids to cytotoxic agents. Sufficient time has now elapsed to pool experience to answer the question about emergence of infections or neoplasia. Of particular relevance at the clinical level is an urgent need to define a correlation between survival of the transplanted organ with some measurement that will reflect optimal immunosuppression and simultaneously predict for the risk of developing complications.

Clearly, much remains to be learned about cyclosporin A. It can, be concluded, however, that this simple substance is a most powerful immunosuppressive agent and is deserving of all the attention it has received to date. Furthermore, cyclosporin A is unique in exerting its effects without the profound suppression of the bone marrow that has, in the past, characterized drugs given to produce immunosuppression.

Finally, there exists the possibility that it may influence a very specific molecular event occurring early in the immunological response to foreign antigen. If this is confirmed, then cyclosporin A has considerable potential as a more selective immunosuppressive agent and may well be a real advance representing the first step in a new generation of agents for clinically effective immunosuppressive therapy.
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Immune Modulation Agents and Their Mechanisms

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With 149 Figures and 127 Tables

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Summary

In adult patients with severe acute aplastic anaemia, primary therapy with CyA alone (n = 6) was compared in a prospective randomised trial to an equivalent amount of this immunosuppressive agent in combination with antilymphocyte serum (n = 6). At minimum follow-up of 36 months no response could be attributed to either regimen. Thus, the use of CyA, either on its own or in combination with antilymphocyte serum, cannot be recommended for this indication.

Introduction

Allogeneic bone marrow transplantation is the preferred treatment for patients under the age of 45 with severe acute aplastic anaemia having a suitable donor [3]. Without this option, supportive care [16] is essential, but anabolic androgens do not significantly alter the course of the disease [3]. The demonstration that antilymphocyte serum or antithymocyte globulin [6, 11] produced variable response rates suggested that in some individuals an immunologic mechanism may be active in pathogenesis. To further explore this possibility the immunosuppressive agent, CyA [14], was randomly given to consecutive patients with severe acute aplastic anaemia, either alone or in combination with antilymphocyte serum.

Materials and methods

Twelve patients with severe acute aplastic anaemia [2] were randomised to receive either CyA alone (n = 6) or the same schedule of this agent with concurrent infusion of antilymphocyte serum (n = 6). The trial was approved by the Ethics Committee of the University of Cape Town and Groote Schuur Hospital, and informed consent obtained from each patient. CyA was given at a loading dose of 25 mg/kg/day in two divided doses for one week, followed thereafter by a daily split schedule, with dosage adjustment using radioimmunoassay (Ciclosporin RIA Kit, Sandoz, Switzerland) [12] to maintain peak levels 4 hours after the dose between 300 and 500 ng/ml and trough levels between 200 and 400 ng/ml. Antilymphocyte serum (Merieux, France) was administered by intravenous infusions over half an hour, at a dose of 15 mg/kg on each of 4 consecutive days. The CyA was continued for a minimum period of 3 months unless undesirable side effects necessitated withdrawal.

Patients who failed to respond after 3 months continued receiving CyA and, in addition, were given oral prednisone (0.5 mg/kg/day) in combination with oral oxymethalone (2 mg/kg/day), the latter being increased to the maximum tolerated dosage. All patients received standard supportive care in the form of red cell and platelet transfusion; appropriate antibiotics were given for infections.

Hematologic response was defined as a rise in platelet count above $50 \times 10^9/\text{l}$, absolute granulocytes above $1 \times 10^9/\text{l}$, and no red cell transfusion requirement [8].

Results

The median age of the patients was 27 years (range 12–49); there were 8 males...
and 4 females. In none was there any ascertainable cause for the aplastic anaemia. The period from diagnosis to commencement of CyA varied from 1 month to 2 years. Three patients were lost to follow-up; one at 2 months and two at 18 months. One patient subsequently received bone marrow from an HLA identical and MLC non-reactive sibling, having initially elected to enter the trial of immunosuppressive therapy. Patients have been followed for a minimum of 36 months.

There was no sustained response in either group.

Two patients responded to therapy during the second phase of the study whilst receiving additional prednisone and oxy-metholone. In one patient the remission was sustained until he was lost to follow-up after 18 months. A second patient achieved a transient response only and died after 18 months of treatment, at which time she was again aplastic.

All patients are accounted for, having either died or been lost to follow-up; median survival was 8 months (3-36 months).

Some degree of toxicity occurred. With CyA administration, an elevation of serum creatinine and diminishing clearance was recorded in 6 of the 12 patients but was reversed following dosage adjustment in all but one, in whom the drug was discontinued. Tremor developed in 2, hirsutism in 1, hypertension in 1, and transient elevation of the serum bilirubin level in 3 patients. The antilymphocyte globulin resulted in serum sickness in one individual, and generalised convulsions occurred in a second patient.

**Discussion**

Severe acute aplastic anaemia may occur as a result of irreversible damage to the haematopoietic stem cell [1] although an indistinguishable syndrome can arise on an immunologic basis [15]. Most established cases do not respond to standard forms of immunosuppressive therapy, including adrenocorticosteroids and additional cyclophosphamide administration [7], but there have been reports of significant improvement following antilymphocyte serum and antilymphocyte globulin [6, 11]. The decision to undertake this trial, evaluating CyA in previously untreated patients with severe acute aplastic anaemia, remains largely empirical. Available evidence for the action of this agent suggests that maximum efficiency would be anticipated at the time when antigen exposure first occurs [9] and this may account for the apparent lack of response in our patients.

The combination with antilymphocyte serum, anticipated to be additive to the functional lesions produced by CyA in abrogating a potentially cellular mechanism in the aplastic patients, was equally unsuccessful. The results of treatment with both antilymphocyte serum and antilymphocyte globulin are variable and may reflect differences in dosage, duration of therapy, quality, and type of protein used [5, 15]. Because of the small number of patients in our series, it is not possible to draw any conclusions about the effect of either this particular product or the schedule used for its administration. However, the dosage was lower than had been reported in some trials, where a more favourable outcome was achieved [4]. Alternatively, the failure to obtain any response may be due to concurrent administration of CyA which modified the protein or its action, thereby rendering it ineffective. The subsequent administration of adrenocorticosteroids and anabolic androgens was included to ensure that any patients with hypoplasia rather than aplasia would not be compromised [13]. This approach would appear to be justified in view of the two patients where a response was documented.

CyA is acknowledged to have a range of toxic effects [10] and patients receiving this agent require scrupulous clinical and biochemical monitoring.

It is concluded from this data that CyA either as a single immunosuppressive agent or when used in combination with antilymphocyte serum has no place in treating patients with severe acute aplastic anaemia. The two responses in the present series are attributable to the subsequent administration of adrenocorticosteroids and anabolic androgens. It can, however, not be discounted that in one of these individuals the antilymphocyte serum may have had a minor or contributing role in the response.
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Cyclosporin A in the treatment of severe acute aplastic anaemia

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SUMMARY. Twelve consecutive adults with severe acute aplastic anaemia, not having a bone marrow transplantation option, were prospectively randomized to receive either cyclosporin A alone or an equivalent amount of this immunosuppressive agent in combination with antilymphocyte serum. The minimum follow-up is 36 months, with half the patients developing nephrotoxicity, which was easily reversible in all but one. No response could be attributed to either regimen. Cyclosporin A does not appear to have a place as primary form of treatment for adults with severe acute aplastic anaemia, either on its own or in combination with antilymphocyte serum.

Bone marrow transplantation is the preferred form of treatment for patients under the age of 40 with severe aplastic anaemia where a suitable donor is available (Storb et al. 1980, 1982; Bortin et al. 1981; Gluckman et al. 1981). In the absence of this option alternative approaches have included supportive care using transfusion of red cells and platelets, with antibiotic administration for infectious episodes (Young, 1981). Anabolic androgens have been prospectively evaluated and shown not to significantly alter the course of the disease (Camitta et al. 1979). Recently, varying response rates have followed the administration of antilymphocyte serum (ALS) or antithymocyte globulin (ATG) (Champlin et al. 1983; Fairhead et al. 1983; Miller et al. 1983; Camitta et al. 1983) to patients with aplastic anaemia, thus supporting the concept that some individuals may have an immunologic mechanism operating. T-lymphocytes are known to play a central role in both immune regulation and the pathogenesis of autoimmune disease and have been shown to produce haematopoietic stimulatory factors (Bacigalupo et al. 1981; Burstein et al. 1982) so that imbalance between helper and suppressor subsets may find expression in marrow aplasia. Such a situation may be reversed by administration of immunosuppressive agents such as cyclosporin A (Stryckmans et al. 1984).

To evaluate a role for cyclosporin A in the treatment of severe aplastic anaemia
consecutive adults, only one of whom had a transplant option, were randomized to receive this agent on its own or in conjunction with antilymphocyte serum.

MATERIALS AND METHODS

Twelve patients who fulfilled the criteria for diagnosis of idiopathic severe aplastic anaemia (Camitta et al. 1976) were randomized to receive either cyclosporin A alone (n=6) or the same schedule of this drug with concurrent infusion of antilymphocyte serum (n=6). The trial was approved by the Ethics Committee of the University of Cape Town and Groote Schuur Hospital, and required informed consent.

Cyclosporin A was given orally at a loading dose of 12.5 mg/kg/d for 1 week, followed thereafter by 6.25 mg/kg/d in two divided doses. Serum levels were monitored weekly by radioimmunoassay (Ciclosporin RIA Kit: Sandoz, Basle, Switzerland) (Randall & Jacobs, 1984). Dosage was subsequently adjusted so that peak levels measured 4 h after the previous dose were maintained between 500 and 500 ng/ml and trough levels between 200 and 400 ng/ml. Antilymphocyte serum (Merleux, Lyon, France) was administered by intravenous infusions over half an hour at a dose of 15 mg/kg on each of four consecutive days (Jansen et al. 1982). The cyclosporin A was continued for a minimum period of 3 months unless undesirable side effects necessitated withdrawal.

Patients who failed to respond after 3 months continued receiving cyclosporin A and, in addition, were given prednisone, at a dose of 0.5 mg/kg/d, and oxymetholone, starting at a dose of 2 mg/kg/d orally and increasing this to a maximally tolerated dose. All patients received standard supportive care in the form of red cell and platelet transfusions; appropriate antibiotics were given for infections.

Haematological response was defined as a rise in platelet count to greater than 50 x 10^9/l, absolute granulocytes above 1 x 10^9/l, and no red cell transfusion requirement.

RESULTS (Table I)

The age of the patients ranged from 12 to 49 years, with a median of 27; there were eight males and four females. In none was there an ascertainable cause for the aplastic anaemia. Three patients were lost to follow-up; one at 2 months (No. 5) and two at 18 months (Nos. 3 and 11) of continuous observation. One patient (No. 12) subsequently received marrow from an HLA identical and MLC non-reactive sibling, having elected to enter the trial of immunosuppressive therapy initially. The period from diagnosis to commencement of cyclosporin A varied from 1 month to 2 years, with all patients meeting the criteria for severe aplastic anaemia on entry to the study. With the previously mentioned exceptions, patients have been followed for a minimum of 36 months.

Two patients responded to therapy during the second phase of the study, following addition of adrenocorticosteroids and anabolic androgens. In one patient (No. 11) the remission was sustained until he was lost to follow-up after 18 months. A second patient (No.
Cyclosporin A in Aplastic Anaemia

Table 1. Data on 12 patients with severe aplastic anaemia treated initially with cyclosporin A alone (n=6) or in combination with antilymphocyte serum (n=6) and, after 3 months, with the addition of adrenocorticosteroids and anabolic androgens

<table>
<thead>
<tr>
<th>Pt No.</th>
<th>Age</th>
<th>Sex</th>
<th>CYA</th>
<th>ALS</th>
<th>Prednisone and/or anabolic androgen</th>
<th>Time from diagnosis to commencing CYA/ALG (months)</th>
<th>Response</th>
<th>Survival (months)</th>
<th>Side effects</th>
<th>Comments</th>
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<tr>
<td>1</td>
<td>28</td>
<td>M</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>8</td>
<td>Renal</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>M</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>-</td>
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<td>26</td>
<td>M</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>5</td>
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<td>-</td>
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<td>F</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>4</td>
<td>+</td>
<td>18</td>
<td>Hypertrichosis, hepatic, renal, hypertension</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>F</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>Hepatic</td>
<td>Lost to follow-up</td>
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<tr>
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<td>31</td>
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<td>+</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>36</td>
<td>Renal</td>
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<tr>
<td>7</td>
<td>28</td>
<td>F</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>4</td>
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</tr>
<tr>
<td>8</td>
<td>24</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>24</td>
<td>-</td>
<td>3</td>
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<tr>
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<td>12</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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<td>49</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>18</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>31</td>
<td>M</td>
<td>+</td>
<td>+</td>
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<td>2</td>
<td>-</td>
<td>3</td>
<td>Renal</td>
<td>Transplanted</td>
</tr>
</tbody>
</table>

4) achieved a transient response only and died after 18 months of treatment, at which time she was again aplastic.

All patients have died or been lost to follow-up and survival in those where analysis is possible ranged from 3 to 36 months, with a median of 8 months; there was no significant difference in the two groups.

Some degree of toxicity occurred. With cyclosporin A administration, deteriorating renal function was demonstrable on biochemical testing in six of the 12 patients, reflected in an elevated serum creatinine and diminishing clearance. In only one of these individuals, however, was it necessary to discontinue the cyclosporin A before the renal toxicity was reversed. Tremor developed in two, hirsutism in one, and hypertension in one. Hepatotoxicity with rising bilirubin level occurred in three patients. The antilymphocyte globulin resulted in serum sickness in one individual, and in a second, generalized convulsions occurred.
Severe acute aplastic anaemia may occur as a consequence of a severely defective haematopoietic stem cell (Boggs & Boggs, 1976), but an indistinguishable clinical and haematological syndrome can arise on an immunologic basis (Thomas & Storb, 1984). Although most cases of severe acute aplastic anaemia do not respond to standard forms of immunosuppressive therapy, such as adrenocorticosteroids and additional cyclophosphamide administration (Criner, 1980), there have been a number of reports showing significant improvement following antilymphocyte serum and antithymocyte globulin administration (Champlin et al. 1983; Fairhead et al. 1983; Miller et al. 1983; Camitta et al. 1983). In this regard it has been suggested that a response to administration of immunosuppressive agents may be predicted on the basis of in vitro testing (Thomas & Storb, 1984; Bacigalupo et al. 1981; Torok-Storb et al. 1984) but, at least using bone marrow, this has not been our experience (Jacobs & Randall, unpublished observation).

The decision to undertake this trial evaluating cyclosporin A in previously untreated patients with severe acute aplastic anaemia remains largely empirical. Available evidence for the action of this agent suggests that maximum efficacy would be anticipated at a time when antigen exposure first occurs (Lafferty et al. 1983) and this may account for the apparent lack of response in this group of patients.

The addition of antilymphocyte serum was designed to include at least one other agent considered to be efficacious in this situation (Champlin et al. 1983; Fairhead et al. 1983). The known effects of ALG (Greco et al. 1983) may be anticipated to be additive with the functional lesions produced by cyclosporin A in abrogating a cellular mechanism in aplastic patients. The results of treatment with both antilymphocyte serum and antithymocyte globulin are variable and this may, in part, be explicable by a number of factors, including dosage, duration of therapy, quality, and type of protein used (Thomas & Storb, 1984; Coiffier et al. 1984). The number of patients tested in our series is small and it is consequently not possible to draw any conclusion about the effect of either this particular product or the schedule used for its administration in the present study. However, the dosage used was lower than has been reported in trials where the outcome was more favourable (Champlin et al. 1983; Fairhead et al. 1983). Failure to obtain any response could have been due to the concurrent administration of cyclosporin A which may have modified the protein or its action, thereby rendering it ineffective. The subsequent administration of adrenocorticosteroids and anabolic androgens was included to ensure that any patients with hypoplasia rather than aplasia would not be compromised (Skarberg et al. 1973). This approach would appear to be justified in view of the two patients where a response was documented.

Cyclosporin A is acknowledged to have a range of toxic effects (Lampacis, 1983) and patients receiving this agent require scrupulous clinical and biochemical monitoring.

It appears that cyclosporin A as either a single immunosuppressive agent or in combination with antilymphocyte serum has no place in treating adults with severe acute aplastic anaemia and that the two responses occurring in this series are attributable to the subsequent administration of adrenocorticosteroids and anabolic androgens. It can, however,
not be discounted that in one of these individuals the antilymphocyte serum may have had a minor or contributing role in the response.

ACKNOWLEDGMENTS

Supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the Medical Research Council and the National Cancer Association. We thank Sandoz Laboratories, Switzerland, for the donation of cyclosporin A. Keren Edwards for help with data collection, Jackie Davies for typing, physicians who referred patients, and the Chief Medical Superintendent of Groote Schuur Hospital for permission to publish.

REFERENCES


PREFORMED LYMPHOCYTOTOXIC ANTIBODIES DISAPPEAR FOLLOWING CYCLOSPORINE THERAPY


HEART TRANSPLANTATION IV, 362-363, 1985

INTRODUCTION
Sensitization in organ transplant recipients leads to difficulty in finding a compatible donor when retransplantation becomes necessary. This study presents our observations in two sensitized patients with heterotopic heart transplants (HHT) in whom lymphocytotoxic antibodies disappeared following the administration of cyclosporine (Cy). Our observations have suggested that Cy may possibly have an effect on B-cell activity in sensitized patients.

Case 1: A 14 year old boy, with no preformed antibodies against a panel of 21 donor lymphocytes covering all major HLAA, B and C loci, underwent HHT for end-stage familial cardiomyopathy. Immunosuppressive therapy consisted of azathioprine (Aza), methylprednisolone (mPred), and rabbit antithymocyte globulin (RATG). During the first three postoperative months he experienced two episodes of severe acute rejection, which were controlled with increased doses of mPred and RATG therapy. Eighteen months after HHT, his exercise tolerance steadily started to decline, requiring anti-failure therapy. The graft showed an advanced degree of coronary arteriosclerosis, and a retransplantation was planned. At this time, a test for T-lymphocytotoxic antibodies in the patient's serum, using the standard NIH microlymphocytotoxicity test, was weakly positive. No previous tests for antibodies had been carried out since the time of HHT and no blood transfusion had been given since the operation. A month later, the antibody titre had increased. The patient's serum was positive against 25% of the same panel cells, and cytotoxicity against half of these cells. It was not possible to relate the antibodies to the first donor's HLA type. Five consecutive potential donors were found to be unacceptable in view of a positive donor-recipient lymphocytotoxic crossmatch. A sixth donor was found, with a negative crossmatch, and the patient underwent an orthotopic heart transplantation (OHT) two years after the HHT, the first graft being left in situ. The reason for choosing this procedure has been reported elsewhere. Until the time of retransplantation the patient was receiving Aza 2.5 mg/kg/day and mPred 0.3 mg/kg/day. Following OHT, immunosuppression consisted of cyclosporine (Cy, 12 mg/kg/day reduced to a maintenance dose of 5 mg/kg/day) and mPred (1 mg/kg/day reduced to 0.25 mg/kg/day at six months). Ten days after Cy therapy was initiated, the titre of the circulating antibodies dropped (15% positive against the panel, with only a 10% cytotoxicity). Subsequently, lymphocytotoxic antibodies were no longer present. The patient did not develop any episodes of acute rejection and remains well 15 months after OHT.

Case 2: This 14 year old patient with dilated cardiomyopathy had no preformed lymphocytotoxic antibodies against a panel of 21 donor lymphocytes when he underwent HHT. Immunosuppression consisted of Aza, mPred and RATG. After several moderate-to-severe acute rejection episodes, Aza was replaced with cyclophosphamide. He experienced no further acute rejection episodes, and remained active and well. By the end of the third postoperative year, the patient's effort tolerance began to decrease because of allograft arteriosclerosis. Six months later he was accepted as a candidate for retransplantation. At that time, weak lymphocytotoxic antibodies were demonstrated in his serum against the standard panel (Figure 1). Antibody titre steadily increased over the next two months (25% positive, with a 75% cytotoxicity). No screening for lymphocytotoxic antibodies had been carried out and no blood transfusion had been given since the HHT. It was not possible to confirm the relationship between these antibodies and the original donor's HLA type. Three donor hearts could not be used because of positive lymphocyte crossmatch. Cy (10 mg/kg/day) was added to the patient's regimen and the dosage was reduced over the next three months. During that period, no cytotoxic antibodies were detected. However, when whole blood Cy trough levels fell below 100 ng/ml, the lymphocytotoxic antibodies reappeared. Cy dosage was increased and again, ten days later no antibodies could be detected. Under Cy therapy the patient had negative crossmatches with four potential donors, but all the allografts were used in other patients who were more acutely ill. Eventually, the patient underwent a OHT, four years after his first operation. The procedure was the same as the one performed in Case 1. Immunosuppressive therapy consisted of Cy and mPred. The patient experienced one episode of moderate acute rejection, but otherwise has made uneventful progress, and remains well one year after the second transplantation. Since this second operation, no antibodies have been detected in his serum.
FIGURE 1: Case 2: Loss of lymphocytotoxic antibodies under Cy regimen. Temporary reappearance of antibodies when the dosage (... of Cy was reduced and Cy trough levels (...) fell below the therapeutic range. Following increased Cy dosage, the antibodies again disappeared.

(kt = second heart transplant procedure. The cross-hatched columns indicate the percentage of panel cells against which lymphocytotoxic antibodies were present.)

DISCUSSION
The immunosuppressive mechanism of Cy remains uncertain. The drug is known to inhibit the activation of the allograft-specific T-cell response, and to prevent activation of helper T-cells by inhibiting the production of interleukins by the inducer T-cell and the macrophage. Cy appears to spare suppressor T-cells. In vitro, there is evidence that Cy suppresses antibody synthesis by B-cells. In vivo, inhibition of T-dependent B-cells has been shown in the mouse. The administration of Cy in the two patients reported here coincided with the disappearance of T-lymphocytotoxic antibodies from their sera; in one patient (Case 2), the presence of antibodies appeared to be Cy dose-dependent. The other patient received increased doses of mPred during the period when the antibodies disappeared. Thus, these observations need to be interpreted with caution. Neither patient was highly sensitized, and panel reactive antibodies of the strength seen in these two cases may vary unpredictably. However, it is unusual for antibodies, even when relatively weak, to disappear quickly, and the association of this phenomenon with the introduction of Cy is of interest. In both cases, the same panel of 21 lymphocytes was used; changes in panel cells therefore can not account for the disappearance of the antibodies, and neither patient had received a blood transfusion since the HHT, which might have altered his antibody status. In neither case have antibodies been detected subsequently, despite the fact that the chronically rejected first transplanted heart, which might have been the source of the antibodies, remained in situ.

The presence of T-lymphocytotoxic antibodies in a patient awaiting transplantation reduces the pool of compatible donors. Although our observation requires confirmation in a large group of highly sensitized patients, it suggests that pretreatment with Cy may result in the disappearance of preformed antibodies, and may therefore increase the donor pool available to sensitized patients awaiting heart transplantation, where a delay may result in death, and in those awaiting renal transplantation, where it could allow earlier transplantation and reduce the pressure on hemodialysis facilities.

REFERENCES
Reactivity of Pretransplant Cytotoxic Antibodies to a Selected HLA Panel Is Not Influenced by Cyclosporin A, With or Without Plasma Exchange

Charles R. Swanepoel, Michael J.D. Cassidy, Margaret May, Machteld Oudshoorn, Ernette du Toit, Lucille Wood, and Peter Jacobs

The University of Cape Town Leukaemia Centre and the Departments of Haematology (L.W., P.J.) and Medicine (Renal Unit), (C.R.S., M.J.D.C., M.M.) Groote Schuur Hospital, and the Provincial Laboratory for Tissue Immunology (M.O., E.d.T.), Observatory, South Africa

The influence of 6 weeks of cyclosporin A (CYA), followed by a further 6 weeks of this agent in combination with plasma exchange (PE), was defined on panel reactivity and titre of preformed cytotoxic antibodies in stable patients on haemodialysis awaiting renal transplantation. Nine individuals with antibodies to 30% or more of the donor panel were entered into the study, but three failed to complete the programme. Comprehensive data are available on the remaining six patients, one of whom was studied twice. The pattern of reactivity to the panel was unaltered, but antibody titres were significantly reduced (P < 0.006). In one patient, a lymphocytotoxic crossmatch performed between the patient and an HLA-haploidentical sibling in the last week of the trial was positive, suggesting that neither procedure was successful in removing an antibody directed against the HLA antigens of a family donor. Three cadaver renal transplants were performed during or after the trial, and while two of the grafts were unsuccessful, one survives at 60 months, with good function. From these data it is concluded that neither cyclosporin A nor its combination with plasma exchange have any effect on the panel reactivity of preformed cytotoxic antibodies, whereas the titres were significantly reduced. An important and serious side effect of the apheresis procedure was the loss of fistulae in two patients.

Key words: panel reactivity, renal transplantation, apheresis procedure

INTRODUCTION

Sensitization to the major histocompatibility complex antigens may preclude patients with renal failure from undergoing kidney transplantation. These individuals block dialysis stations and, where resources are limited, will deny otherwise suitable patients access to both this procedure and subsequent renal transplantation. Such sensitization may occur as a natural phenomenon [1], following pregnancy [2], transplantation, or blood transfusion [3], although the availability of recombinant human erythropoietin [4] may replace the latter and thereby abolish this form of immunisation.

A number of options have been evaluated for the removal of antibodies once they have been formed, in an attempt to salvage patients and render them suitable for transplantation, including plasma exchange and steroids with or without azathioprine, cyclophosphamide, or cyclosporin A (CYA) [5–8]. Despite success having been limited to those patients where cytotoxicity was directed against one or two of the major HLA antigens [5,6], it seemed important enough to prospectively re-examine the effect of CYA on its own when combined with plasma exchange (PE) on both the spectrum of reactivity of the preformed cytotoxic antibodies and their titre.

PATIENTS AND METHODS

Entry required the presence of preformed cytotoxic antibodies reacting to more than 30% of a donor panel comprising 30 cells selected to represent the major HLA phenotypes. All patients who had absence of renal function, were red cell transfusion independent, free of sepsis, and had no history of prior liver disease. The protocol was approved by the University of Cape Town and Groote Schuur Hospital Ethics and Research Committee, and entry required informed consent. Patients underwent standard haemodialysis three times a week and in the

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first 6 weeks received only CYA at a dose of 4 mg/kg twice a day. Thereafter, dosage was adjusted to maintain whole blood trough levels between 200 to 400 ng/mL [9]. In a second phase, the CYA was continued and isovolaemic PE, using the Cobe Centry parallel-membrane filtration unit, preceded the haemodialysis. These procedures were carried out twice a week in the first 2 weeks and weekly thereafter. Using nomograms, 1.5 × the calculated plasma volume was replaced with 4% salt-free human albumin in saline, to which was added 6 mL of 10% calcium gluconate/L.

Laboratory investigations, which were carried out at entry to the trial and then at the end of each 6 week phase, included full blood count, measurement of serum calcium, and inorganic phosphorus and alkaline phosphatase levels. Serum parathyroid hormone (PTH) could only be measured in two of the patients.

The level of cytotoxicity, defined as a percentage of panel reactivity, was measured using lymphocytes from 30 HLA-typed donors. The titre of cytotoxic HLA-antibodies was determined by allowing serial dilutions of the patient serum to react with four individuals who had been selected from the panel by their having maximum reactivity with the undiluted patient serum. Subsequent antibody titrations were always performed against the same set of four cells. Sera were tested for IgG and IgM antibodies, using the reducing agent, dithiothreitol (DTT) [10]. Tests were carried out on a control group of seven patients on maintenance dialysis, also having > 30% panel reactivity and who were not treated with either CYA or PE.

STATISTICAL ANALYSIS

The Student t test was used to determine the significance of changes in panel reactivity, calcium, inorganic phosphorus, and alkaline phosphatase levels. The Mann-Whitney U test was used to check the significance of changes in titres of antibody.

RESULTS

Nine patients entered the trial (Table I), but three were withdrawn at 4, 7, and 4 weeks, respectively; one underwent renal transplantation; in the second, the fistula clotted and the patient developed sepsis and required transfusion; and the third, also because of blood transfusion requirements. A total of two patients lost their fistulas following PE. Seven studies were undertaken in six patients. There were no changes in the full blood count, serum calcium, or inorganic phosphorus levels. However, changes in the serum alkaline phosphatase were significant (P < 0.05) when pretreatment levels were compared to 6 weeks of CYA and PE. Parathyroid hormone levels were determined in two patients, and in both, the levels were above the normal range throughout the study period.

In the control group (n = 7), there were no changes in the percentage panel reactivity or in the HLA-antibody titre levels. In the patients, there was, similarly, no change in the percentage panel reactivity in the 3 month control period or in the period during which they were treated with CYA, with or without PE (Fig. 1). However, in two patients, there was a 73% and 46% drop over the 12 weeks of the study (Fig. 2), and both of these individuals underwent renal transplantation after completing the 12 weeks trial period.

The majority of the cases were initially sensitized by transplantation, and apart from patient no. 3, all had multispecific antibodies; the antibody class determined in five was predominantly IgG. Measurement of cytotoxic antibody titres showed no change in the 12 weeks prior

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>Diagnosis</th>
<th>Kidney failure time (years)</th>
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<th>TX</th>
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<th>HLA specificity</th>
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<td>2</td>
<td>N.D.</td>
<td>Multi</td>
</tr>
</tbody>
</table>

Kidney failure: RPGN = rapidly progressive glomerulonephritis, CGN = capillary glomerulonephritis, COR. NEC. = cortical necrosis, MCGN = mesangio-capillary glomerulonephritis, SLE = systemic lupus erythematosus, ANAL = analgesic nephropathy, Time = time from when first dialysis was necessary until trial commenced, TRANS = blood transfusions after the commencement of the trial, TX = number of renal transplants prior to the start of the trial; ND = not done.
to study, but there was a significant fall after CYA and PE ($P < 0.006$) (Fig. 3).

Three patients were transplanted either during or soon after completion of the study (Table II). One patient, no. 3, with good kidney function survives 60 months after allografting, with a serum creatinine of 152 $\mu$mol/L; this was the only patient in the group who had antibody specificity restricted to the HLA antigen A24 and showed a 46% reduction in panel reactivity in response to the treatment (Fig. 2).

The lymphotoxicity cross-matching test was performed between one of the study patients and his HLA-haploidentical sibling during the last week of the study, while receiving CYA and on PE, and was shown to be positive.

**DISCUSSION**

Although the aim of the study was not to investigate the reason for the development of cytotoxic antibodies, where much larger numbers of patients would have been required, it nevertheless appears unlikely that they were naturally occurring [1]. Thus, in the present series, the majority of these reacted weakly with B-lymphocytes, but strongly and predominantly with T-cells. This finding contrasts with the rare, naturally occurring forms that have weak reactivity directed only against B-lymphocytes. Furthermore, while recognising a role for pregnancy in isoinmunisation, this mechanism could be confidently invoked only in one of the six females studied.

In the remainder, we consider renal transplant to have been the likely precipitating mechanism, with transfusions playing a complementary role.

Irrespective of the causative mechanism, it is notable that successful removal of HLA antibodies can be achieved using combined PE and immunosuppression [5,6]. In those cases, antibodies were directed only at one or two antigens, which again contrasts to the multiple specificity of antibodies in the current series. In our experience, percentage panel reactivity was unaltered by either manoeuvre, while titres were significantly reduced.

<table>
<thead>
<tr>
<th>TABLE II: Details of the Three Patients Transplanted</th>
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<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
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</tr>
<tr>
<td>1</td>
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<tr>
<td>3</td>
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<tr>
<td>7</td>
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</tbody>
</table>

A = time spent waiting for a transplant prior to the trial; B = time spent waiting for a transplant after the start of the trial; Tx = transplantation; NF = never functioned; GF = good function.
Another possible explanation for the difference in results may well lie in the lesser intensity of our PE programme. Furthermore, CYA was used without additional steroids or other immunosuppressive agents. Of interest is a case report in which intensive PE did not alter antibody titres. This was probably related to repeated blood transfusions given to the patient during the course of study [7].

It has been suggested that CYA combined with PE has no influence on reducing sensitization [8]. It is possible that this combination may only be effective when the antibodies are specifically directed against one or two HLA antigens. In this regard, of the three patients in the present series who were transplanted, one survivor had antibodies restricted to HLA-A24 and the percentage panel reactivity of her antibodies showed a marked change from the level at entry to that after 12 weeks on combination CYA and PE. The remaining two patients who were transplanted never established graft function, and both had multispecific antibodies. It is of note that the three transplanted patients had waited unsuccessfully for an average of 24 months for a cadaver kidney before the start of the trial, whereas they underwent allografting on an average of 2.8 months after the study was initiated. It may well be that the combination of CYA and PE would have much greater benefit if initiated immediately when the problem is identified and, once the titre is reduced, the patient proceeds immediately to renal transplantation. Additionally, better results might be anticipated if patients were entered on the combination of CYA and PE when antibodies are directed against a single HLA antigen.

As part of a wider study examining the complications of PE, alkaline phosphatase was tested and found to fall significantly during the course of this investigation. This may be attributable to the mass removal of the enzyme by the exchange procedure, since it is unlikely to represent healing of osteodystrophy as serum calcium and PTH levels remained abnormal. It is noticeable that the opposite effect on this enzyme has been reported with CYA administration to patients undergoing renal allografts [11,12], where there was an increase in osteoblastic activity with resultant elevation in the plasma levels; we are unable to explain this discrepancy.

The failure to complete the study resulted when the patients proceeded to transplantation, required blood transfusions, or developed sepsis. Two patients lost their arteriovenous fistulae as a direct consequence of the PE procedure. This is a serious complication since the loss of a fistula may shorten the lifespan of a patient on dialysis.
It is concluded that the combination of CYA and PE significantly reduces antibody titre without influencing overall reactivity to a panel of cells selected to contain the most commonly occurring HLA antigens. Furthermore, this regimen was unable to reduce antibodies against family donor HLA antigens in the one case studied. Only a single patient has a functioning cadaver transplant 60 months after the trial. On the basis of these results, CYA, with or without PE, cannot be recommended as an effective means of altering multispecific cytotoxic antibodies or therefore as a basis for rendering renal graft function more successful in these individuals. However, the removal of anti-HLA antibodies remains a challenge for the future and the use of more selective techniques [13], perhaps including extracorporeal staphylococal protein A immunoabsorption, may be a practical alternative [14].

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We thank Gail Randall for cyclosporin A assays, Douglas Bell for measuring the percentage panel reactivity and HLA antibody titres, and Jackie Davies for help with preparation of the manuscript and its typing.

REFERENCES


Cyclosporin A pretreatment of both donor and recipient undergoing allogeneic bone marrow transplantation

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51 patients received allogeneic marrow from histocompatible and MLR non-reactive siblings. Post-transplantation methotrexate was associated with acute refractory graft-versus-host disease (GVHD) in 10/23 (43%) and caused death in 6. When cyclosporin was substituted for the methotrexate fulminating GVHD occurred in 2/28 (28%): both responded to methylprednisolone. When both donor and recipient were pretreated with cyclosporin GVHD of only mild degree developed in 6/20 (30%): 2 responded rapidly to methylprednisolone and in the remaining 4 mild cutaneous lesions persisted. The latter regimen was associated with no donor morbidity and approximately 50% of the recipients had easily reversible renal dysfunction. Thus, cyclosporin A appears to marginally reduce the incidence of acute GVHD and to facilitate response when additional methylprednisolone is required: additional pretreatment of the donor appears to reduce the severity of the acute syndrome. In none of these regimens was a beneficial effect observed on de novo GVHD.

Accepted for publication May 9, 1985

Allogeneic bone marrow transplantation is the preferred form of treatment for severe acute aplastic anaemia (1), is increasingly used for patients with both myeloblastic (2) and lymphoblastic (3) leukaemia, and is being evaluated in a variety of other diseases (4). Despite meticulous matching of siblings at the major histocompatibility complex and their non-reactivity in the mixed lymphocyte culture, both acute (5) and chronic (6) GVHD occur and together constitute a formidable barrier to the more widespread use of marrow grafting.

Attempts to prevent (1, 7) or abrogate (8, 9) GVHD have met with variable success and since the introduction of cyclosporin A its effect on transplantation in general (10) and especially marrow allografting (11) has been encouraging (12). However, while the incidence and severity of acute GVHD with its high morbidity and mortality and chronic GVHD with its relentless progression and mutilating side effects have not been totally eradicated, there appears to be advantage in further exploring the use of this unique immunosuppressive agent. Evaluation of

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Professor Peter Jacobs
different regimens has been directed largely at the recipient (12) and no attention given to donor pretreatment. Since the acute disease may progress to the chronic form and because immunocompetent lymphocytes transferred from donor to recipient in the graft may have a role to play, a pilot study was carried out to test the possibility that additional cyclosporin A pretreatment of the donor may influence the expression of both acute and chronic GVHD following allogeneic bone marrow transplantation.

Material and methods

51 patients underwent allogeneic bone marrow transplantation on a programme approved by the Ethics Committee of the University of Cape Town and after donors and recipients had signed informed consent.

Initially, patients with refractory acute leukaemia were offered transplantation but subsequently only those in consolidated complete remission or having severe acute alastic anaemia were considered eligible. Sibling donors compatible at the major histocompatibility complex and non-reactive with their recipients in the mixed lymphocyte culture were used.

Severe acute aplastic anaemia was defined according to internationally accepted criteria (13) and patients were conditioned with 50 mg/kg of cyclophosphamide by i.v. injection on 4 consecutive days before transplantation (5). The patients with acute leukaemia were treated with a combination of V1P-213, cytosine arabinoside and doxorubicin (14), after which they were conditioned with cyclophosphamide, 60 mg/kg on 2 consecutive days, and 10 Gy total body irradiation at a maximum dose of 7 rads/min given 24 hs after the last dose of conditioning drug. All patients were managed in reverse isolation, received hyperalimentation and such antibiotic and allogeneic component support as was appropriate.

Three different immunosuppressive regimens were evaluated in the course of this study. In Group 1 (n = 23) cyclosporin A was not available and, following transplantation, patients received only methotrexate (5). In Group 2 (n = 8) cyclosporin A was commenced the day before infusion of the graft at an oral dose of 12.5 mg/kg twice a day for a week and then 6.25 mg/kg twice daily for the next week, after which adjustments were made to maintain plasma concentrations between 250 and 500 ng/ml by radioimmunoassay (15) at least once a week for the entire duration that patients received this immunosuppressive drug. In Group 3 (n = 20) both donor and recipient commenced cyclosporin A on the same dosage schedule 2 weeks before transplantation; patients remained on this agent for the same duration as those in Group 2. At all times plasma concentrations were monitored and doses were adjusted at least once every week to maintain levels in the same therapeutic range as in Group 2.

Biochemical measurements of renal and hepatic function, as well as electrolyte status and acid-base balance, were measured on alternate days in donors and recipients. Any increase in creatinine or urea, even whilst still in the normal range, was managed initially by small doses of oral furosemide, and failure to immediately reverse this trend required reduction in cyclosporin A dosage. In the donors as well as in the recipients after discontinuation of cyclosporin A, these measurements were continued on follow-up visits until their discharge from clinic.

Acute (5) and chronic (6) GVHD were defined according to established criteria. Both syndromes were treated by continued administration of cyclosporin A; in the acute disease methylprednisolone was added; in the chronic form additional high-dose pulsed methylprednisolone (9) was initially used and this was followed by long-term corticosteroids and azathioprine. The dose of the two drugs was variable and started at 0.5 mg/kg and 2 mg/kg of lean body mass, respectively. The methylprednisolone was gradually reduced, whereas the dose of azathioprine remained relatively constant and while the efficacy of this two-drug combination on the clinical course of the chronic GVHD was constantly reviewed, average duration is presently in excess of 9 months.

Results

Group 1: n = 23
These patients were treated with methotrexate for immunosuppression (5). 10 of the 23 patients (43%) developed classical severe acute GVHD within 6 weeks of transplantation, which was refractory to therapy and caused death in 6 patients. Of the remaining 4 patients, 1 died of Budd-Chiari syndrome, which may have been related to the long period of prior intensive chemotherapy, and 3 progressed to chronic GVHD, of whom 1 is alive with disfiguring cutaneous lesions, 1 died of disseminated tuberculosis without evidence of GVHD, and the
other died following a seizure but with severe skin lesions; autopsy failed to reveal the cause of death.

Chronic GVHD arose de novo, that is, without any prior acute disease, in 2 patients (9%) and has been self-limiting in both.

Group 2: \( n = 8 \)
These patients received cyclosporin A immunosuppression without donor pretreatment. Severe acute GVHD occurred in 2 patients (25%) and in both responded promptly to additional corticosteroids. 1 patient died from leukaemic relapse and the second from a ruptured cerebral aneurysm.

Chronic GVHD without an antecedent acute episode occurred in 3 patients (38%). 1 died from disseminated fungal infection with poorly controlled cutaneous Grade 1 GVHD. The other 2 developed minimal but characteristic skin lesions 3 months after discontinuing cyclosporin A. Both have responded to restarting cyclosporin A in conjunction with a single course of pulsed high-dose methylprednisolone.

TABLE 1
<table>
<thead>
<tr>
<th>Group-versus-host disease in 51 patients.</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Group 1 (( n = 23)) patients received methotrexate as their only immunosuppressant agent, in Group 2 (( n = 8)) recipients received only cyclosporin A from the day preceding transplantation, and in Group 3 (( n = 20)) donors and recipients received cyclosporin A for 14 days before grafting and the recipients continued with this as their only immunosuppressive agent after transplantation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group-versus-host disease</th>
<th>Acute</th>
<th>Acute progressing to chronic</th>
<th>De novo chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (( n = 23))</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2 (( n = 8))</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3 (( n = 20))</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

Although the incidence of acute GVHD in the three groups (see Material and Methods) was respectively 43%, 25%, and 30%, these results are not statistically different \(( p > 0.05)\) \((24)\). However, in no patient receiving cyclosporin A did the acute disease progress to chronic GVHD and, furthermore, it was easier to control or reverse the acute clinical syndrome with the addition of corticosteroids.

TABLE 2
Analysis of 51 patients undergoing allogeneic bone marrow transplantation

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (( n = 23))</th>
<th>Group 2 (( n = 8))</th>
<th>Group 3 (( n = 20))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months)</td>
<td>20</td>
<td>17</td>
<td>21.5</td>
</tr>
<tr>
<td>Range (yr)</td>
<td>6-60</td>
<td>6-30</td>
<td>10-46</td>
</tr>
<tr>
<td>Interval from diagnosis to transplantation</td>
<td>3</td>
<td>3.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Range (months)</td>
<td>1-34</td>
<td>1.14</td>
<td>1 month - 8 yr</td>
</tr>
<tr>
<td>Follow-up of living patients</td>
<td>67</td>
<td>26.5</td>
<td>14</td>
</tr>
<tr>
<td>Median (months)</td>
<td>48-98</td>
<td>26-27</td>
<td>4-19</td>
</tr>
<tr>
<td>Diagnosis (Dead)</td>
<td>Aplastic anaemia 13 (10)</td>
<td>6 (5)</td>
<td>10 (5)</td>
</tr>
<tr>
<td>Refractory leukaemia</td>
<td>5 (5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Remission leukaemia</td>
<td>3 ALL, 2 AML (4)</td>
<td>1 ALL, 1 AML (1)</td>
<td>2 ALL, 6 AML (7)</td>
</tr>
<tr>
<td>Chronic granulocytic leukaemia</td>
<td>-</td>
<td>-</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>-</td>
<td>-</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Immediate cause death Infection</td>
<td>Aplastic anaemia 8</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Refractory leukaemia</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Remission leukaemia</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Chronic granulocytic leukaemia</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Haemorrhage</td>
<td>Aplastic anaemia 2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Refractory leukaemia</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Remission leukaemia</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Chronic granulocytic leukaemia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>Aplastic anaemia</td>
<td>-</td>
<td>1 unexplained cardio-myopathy</td>
</tr>
<tr>
<td>Refractory leukaemia</td>
<td>1 Budd-Chiari syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remission leukaemia</td>
<td>1 relapse 1 acute cholelithiasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic granulocytic leukaemia</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hurler syndrome</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The three groups, which were treated sequentially, received alternative forms of immunosuppressive therapy (see Material and Methods).
Group 3: n = 20
These patients and their donors were pretreated with cyclosporin A for 2 weeks before transplantation. 6 patients developed early GVHD 30%. In 2 this was mild and responded promptly and completely to corticosteroid therapy; 1 patient subsequently died from massive gastrointestinal tract haemorrhage and the second patient is currently well. In 4 further patients the disease was also mild, being defined as Grade 1 cutaneous changes, diarrhoea less than 500 ml in 24 h, and without abnormality on liver biopsy. However, in none of these patients could this clinical syndrome be reversed on therapy and all died within 3 months; 3 from infection and 1 from massive haemorrhage from a duodenal ulcer.

Chronic GVHD occurred de novo in 2 patients (10%) after they had discontinued their cyclosporin A and both have responded to one pulse of high-dose methylprednisolone.

Engraftment and Survival
Engraftment is defined as the time taken from transplantation to reach a granulocyte count of $0.5 \times 10^9/l$ or a platelet count of $25 \times 10^9/l$. These findings are substantiated by a bone marrow aspiration and trephine biopsy which showed greater than 15% of haematopoietic cell repopulation. The median time to achieve these criteria for the three groups was 21 (range 9–26), 14 (range 6–33), and 13 (range 7–16) d respectively.

Actuarially predicted survival curves (Figure 1) show little difference for median survival, with a non-significantly higher plateau for patients in Group 3.

Toxicity
In none of the donors did biochemical tests of renal function rise outside the normal range during the 2 week period of cyclosporin A administration. The donors were all fully reassessed, including biochemical measurements, before discharge and again within 1 month after discontinuing cyclosporin A therapy. Furthermore, in a separate series of individuals with severe acute aplastic anaemia without a transplant option, cyclosporin A was used as the primary form of immunsuppression either singly or in combination with antilymphocyte globulin; in 12 individuals monthly follow-up for an average period presently in excess of 9 months has shown no late disturbances in biochemical tests of renal or hepatic function.
In recipients receiving cyclosporin A, transient elevation of blood urea and creatinine was seen in 50% of the patients in Group 2 and 3. Any increment in these biochemical measurements, despite their remaining within the normal range, was managed by treating the patient with 40 mg furosemide daily or twice a day. Failure to immediately reverse the trend required reduction in the dose of cyclosporin A being given. No examples of progressive deterioration or irreversible dysfunction were observed using this approach.

Discussion

In Group 1 patients underwent allogeneic bone marrow transplantation before cyclosporin A was available and methotrexate was used as the only post-transplantation immunosuppressive agent (5). The incidence of severe GVHD was 43%, and in 6 out of 10 patients was directly contributory to their death. In a further 7 patients who died as a consequence of fulminating bacterial infection, although without the classical cutaneous or hepatic stigmata of this syndrome, it may have played a subsidiary or contributory role. However, in these individuals clear separation from the underlying consequences of their primary disease, prior chemotherapy, cyclophosphamide conditioning or radiotherapy was not possible. Of these 10 patients in Group 1 who had classical severe GVHD 3 of the survivors progressed to extensive cutaneous lesions which, although they could be controlled with corticosteroids and azathioprine therapy, created a major and seriously disfiguring complication. The single survivor in this group, now beyond 3 yr, has noted slow reversal of the skin lesions at a time when he is off all therapy.

In Groups 2 and 3 cyclosporin A replaced methotrexate. The novel fungal metabolite is attractive, particularly following allografting, since it is free of myelosuppressive effects as well as the metabolic consequences that accompany prolonged administration of corticosteroids. Its major acute side effects are related to predictable but reversible changes in renal function and, less frequently, to alterations in biochemical tests of liver function. It is not lympholytic, is thought to influence an early step in the immune response, probably by interfering with T-lymphocyte action and, as a result, blocking the generation of effector cells by preventing the recognition of foreign or transplanted antigen (16). Available evidence suggests that cyclosporin A should be administered to the patient before infusion of the graft, but despite testing a number of different schedules no apparent advantage was evident for one over the other (12).

In Group 2, cyclosporin A was therefore commenced at least 24 h before transplantation, and while the numbers are small the incidence of severe acute GVHD was reduced; 25% as compared to 43% in Group 1. A second and more striking feature was the ease with which diarrhoea and deteriorating renal and liver function were reversed in both patients with high-dose steroids (9), although the skin changes responded more slowly. One other observation of interest was the emergence of de novo chronic GVHD in 3 patients (37%), 2 of whom had certainly discontinued their cyclosporin A in the 8 wk before the first mucocutaneous lesions appeared. Here again, resolution occurred within 2 wk of restarting the immunosuppressive drugs (8) and this contrasts with our experience in methotrexate-treated patients. 2 patients died from fungal infection, and while this might be the consequence of immunosuppression resulting from cyclosporin A therapy, its contribution cannot confidently be separated from the effects of the underlying disease, cyclophosphamide conditioning, or possibly even radiotherapy (17).

In Group 3 both the recipient and the donor were pretreated with cyclosporin A on the basis of experimental studies (18) when such a modification to the schedule further diminished the incidence of severe acute GVHD over a matched group of rabbits where only recipients received this agent from the time of allografting. Whilst recognising that the prevailing concept for the action of cyclosporin A requires only its physical presence to impair T-lymphocyte help, there is
no proof that this is its only or even major action. Alternatively, it is not clear whether a longer period of time may not be necessary for in vivo exposure of the donor cells to the immunosuppressive agents to achieve maximum effect. The latter theoretical possibility appears to find support in the mild GVHD occurring in 6 of the 20 patients being transplanted on this schedule and its immediate reversal in 2 upon addition of prednisone. In the other 4 individuals the onset of clinical course was indistinguishable from the 2 who responded, but their cutaneous lesions progressed despite addition of corticosteroids and without ever developing more than 500 ml of diarrhoea in 24 h or abnormalities in liver function or changes on biopsy. Death occurred in these 4 patients due to disseminated aspergillus in 2, bacterial infection in 1 and massive haemorrhage from a duodenal ulcer in 1. Although only skin lesions were present as objective evidence for acute GVHD, this observation is subject to the same reservation that applies to our patients in Group 1: thus, while expression of the clinical syndrome could have been modified by cyclosporin A, it may nevertheless have been present and played a subsidiary or contributory role to the eventual demise of the patients. In 2 additional patients the chronic cutaneous form of GVHD became evident only after cyclosporin A had been discontinued. This pattern of response is similar to the 2 patients in Group 2 whose skin lesion also appeared only after therapeutic levels were no longer present in the plasma. Both patients responded dramatically to high-dose methylprednisolone but are left with areas of increased skin pigmentation.

Since the experience in the three groups was accumulated chronologically, a number of further questions require consideration. Firstly, the patients are not absolutely matched since those in Group 1 contained a number of individuals with refractory leukaemia, whereas this indication for transplantation had been discontinued by the time patients were entered into Group 2 or Group 3 of the study. A second point to emphasize is that the schedule for cyclosporin A administration to the recipient differs between Group 2 and Group 3, with patients in the latter group receiving 14 d pretreatment as opposed to only 1 d in Group 2. It is therefore possible that the duration of recipient pretreatment may account for the apparently superior results seen in Group 3. In this regard, it should be noted that the remainder of the transplantation procedure remains unchanged during the course of these studies. To define the role of donor treatment a prospective randomised trial is now in progress comparing 14 d of cyclosporin A administration before allografting to recipient only with outcome when both donor and recipient receive the identical schedule.

The time to achieve engraftment appears shorter in the two groups receiving cyclosporin A, although the difference between patients in Group 2 and Group 3 is not significant. This shortening in the time taken to engraftment is consistent with other experience (19).

The overall results of bone marrow transplantation in this series are less satisfactory than reported by others, and in this regard infection played a major role in morbidity and mortality. The reason for this is that the data reported are derived from our early transplant figures when many of the patients were referred in less good clinical condition than would now be required for entry on to the allograft programme. In addition, the poor results in aplastic anaemia are clearly related to the extensive prior transfusion with sensitization that had taken place in this group of patients and which would currently exclude referred individuals for grafting. Interstitial pneumonia was approximately equally distributed between the groups but accounted for less than 20% of the infectious deaths.

In a programme where donors are exposed to the additional risk of cyclosporin A over and above general anaesthetic and the unpleasant procedure of bone marrow collection, it is essential that side effects be reduced as far as possible (20–23). In our experience prompt attention to any changes in biochemical tests of renal function avoided nephrotoxicity (17). In addition cyclosporin A administration as the primary form of treatment to patients with severe acute
 aplastic anaemia lacking the option for marrow transplantation has shown transient and easily reversible nephrotoxicity; the average period of follow-up is presently in excess of 9 months and neither persistence nor late development of abnormalities in biochemical tests of renal or hepatic function have emerged (Jacobs et al, unpublished observation).

It is concluded that cyclosporin A may have a role to play in bone marrow transplantation. When it is given only to recipients as the sole immunosuppressive agent there is an apparent decrease in the incidence and severity of acute GVHD. These schedules are not completely effective in preventing the acute syndrome but, should this occur, it is easier to control with methylprednisolone. This was not our experience when methotrexate was used. Although it appears that cyclosporin A has a major effect on T-lymphocyte helper function, it is theoretically possible that a longer period of donor cell exposure may be necessary. This hypothesis was tested by pretreating donor and recipient with cyclosporin A. In these preliminary observations, it appears that marginal reduction in the incidence of acute GVHD may be possible but more strikingly is the apparent decrease in the severity of this syndrome. Furthermore, it also appears as though the number of patients progressing from acute to chronic GVHD may be reduced, whereas de novo chronic GVHD is unaffected. These results suggest that such a schedule of donor pretreatment may warrant further evaluation.

Acknowledgements

This study was supported by the University of Cape Town Leukaemia Centre and Staff Research Fund, the Medical Research Council and the National Cancer Association. The cyclosporin A used was generously donated by Sandoz Laboratories, Switzerland.

I thank Gail Randall and Linda Eglinton for cyclosporin A assay, Keren Edwards and Jackie Davies for research and secretarial assistance, and Chief Professional Nurse Lucille Wood for help with patient care. I express appreciation to colleagues who have referred patients and participated in management, particularly Dr. D. W. Dubovsky, Dr. Christina Geddes, Dr. L. M. Kernoff, and Dr. Ingrid le Roux. Dr. Ernette du Toit and her staff carried out the tissue typing. Thanks are due to registrars and nursing staff from the Transplantation Unit for expert care, Dr. Helen S. King for radiotherapy, and the Chief Medical Superintendent of Groote Schuur Hospital for permission to publish.

References


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15. SEPARATION AND CRYOPRESERVATION OF HAEMATOPOIETIC STEM CELLS FROM HUMAN CADAVER BONE MARROW

S. Horak, L. Wood, P. Jacobs

The University of Cape Town Leukaemia Centre and the Department of Haematology, Groote Schuur Hospital

The normal discoid erythrocyte deformed rapidly during storage and virtually disappeared by Day 9 (0.12%). At this stage the following cells were present: Type B discocytes, Type IA, IB and IC echinocytes, one group of Type II echinocytes, and Type IIIA and IIIB echinocytes. By Day 21 the only red cell types remaining were Type IC, II, IIIA and IIIB echinocytes, Type I and II spherocytoids and echinocytes.

REFERENCES

17. THE EFFECT OF CYCLOSPORIN ON HUMAN LYMPHOCYTES IN VITRO

C. van Eeden, S. Horak, P. Jacobs

The University of Cape Town Leukaemia Centre and the Department of Haematology, Groote Schuur Hospital

Cyclosporin is a novel immunosuppressive agent widely used in widespread clinical use. It acts by impairing the ability of T-helper lymphocytes to release lymphokines, including interleukin 2, thus to respond to appropriate antigenic stimulation. Since therapy is controlled by monitoring whole blood radioimmunoassay, it is a study undertaken to relate these levels to immune suppression, reflected in lymphocyte response to mitogens and alloantigen. Technically, human peripheral mononuclear cells were separated by density gradient centrifugation, washed twice in tissue culture medium, and a suspension of 1 x 10^6/ml cells prepared. Dose and time response curves were determined for cyclosporin concentrations ranging from 0.5-2400 ng/well, using assays calibrated to obtain optimal response to different mitogens and alloantigen. Profound suppression of H² incorporation into DNA under these conditions occurred at a concentration of 100 ng/well: equivalent to a plasma level of 2000 ng/ml. Time-course experiments demonstrated this effect to be maximal when cyclosporin was added to the cultures up to 6 hours following exposure to the antigens. It is therefore concluded that cyclosporin is capable of suppressing lymphocyte function at therapeutic concentrations and that this effect is critically time-dependent. Supported by the University of Cape Town Leukaemia Centre and Staff Research (Cancer) Fund, the National Cancer Association and Medical Research Council.

18. PROBLEMS ENCOUNTERED BY A MEDICAL TECHNOLOGIST DEALING WITH VETERINARY HAEMATOLOGY

A. van Heerden

Faculty of Veterinary science, University of Pretoria.

Due to the current small number of veterinary clinical laboratories in South Africa, both within the industry and in the field of haematology. However, many have realized that there are significant differences between the Haematology of man and that of animals. These differences often lead to confusion. I intend to illustrate the most important interspecies variations in the morphology and proportions of the different types of leucocytes. In addition I will discuss and illustrate the most common blood parasites to be found in our domestic animals.
CICLOSPORINE IN HAEMATOLOGIC PERSPECTIVE

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Immunosuppressive drugs play an important role in the management of autoimmune diseases and are crucial for graft survival in organ transplantation. Cytotoxic agents are limited by varying degrees of myelosuppression and corticosteroids by widespread metabolic complications. Attempts to dissociate these unwanted side effects from the desired modulation of humoral and cell-mediated mechanisms led to the development of ciclosporine. This fungal undecapeptide directly impairs, in a dose-dependent fashion, the function of CD4 or T-helper lymphocytes, with important consequences throughout the immune system, leading to a profound suppressive effect.

This unusual molecule favours engraftment following bone marrow transplantation, but used on its own has not fulfilled its early promise in abolishing acute graft-versus-host disease (GVHD) and is currently combined with other agents for this purpose. In broader haematologic context, its administration improves the outcome in some patients with aplastic anaemia and pure red cell aplasia, and its role is being evaluated in other immune disorders. Toxicity is primarily the impairment of renal and hepatic function, with attempts currently in progress, therefore, to improve monitoring and, in addition, to develop other analogues. It is concluded that the use of ciclosporine is well established in haematology, but in marrow allografting it remains less effective than in transplantation of solid organs.

KEY WORDS: Ciclosporine, immunosuppression, graft vs. host disease, aplastic anaemia.

INTRODUCTION AND HISTORICAL BACKGROUND

The demonstration in dogs that survival of renal homografts could be prolonged following the administration of 6-mercaptopurine marked the beginning of an era examining immunosuppression in transplantation. The early work using conventional cytotoxic drugs was characterised by substantial side effects, notably in the bone marrow, with consequent granulocytopenia and a high infection rate. Attempts were made to dissociate therapeutic benefit from myelotoxicity, with the prototype substance being ciclosporine, a cyclic undecapeptide that was isolated from certain strains of Fungi imperfecti. Initial claims for a relative lack of marrow suppression at clinically effective doses were confirmed and this feature became the basis for its use in transplantation. The agent was subsequently successfully employed in managing a variety of autoimmune conditions, parasitic infections and more recently, at a molecular level, to modulate the behaviour of cytotoxic drugs in tumour cells. Not surprisingly, an extensive literature has accumulated and the present review describes the pharmacology and mechanisms of action as the basis for

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Supported by the University of Cape Town Leukaemia Centre and Staff Research (Foote and Cancer) Fund, the Gwendoline Moore Trust, the Medical Research Council, the National Cancer Association, and the Michael Chanani, Kaliski and M.A. Richardson Bequests.
toxicity, which can be reduced by careful monitoring. The recommended practice is to adjust dosage based on whole blood levels to compensate for variable absorption from the gastrointestinal tract. This approach does not take into account the relative role of the parent substance as opposed to metabolites and represents a compromise since it is not known how best to directly measure the total suppressive effects on the immune system.

High performance liquid chromatography (HPLC) is a well established technique which measures individually the parent substance and the various metabolites. This can be automated so that many samples can be determined with accuracy and precision, although the cost is high. This method may have advantages where specific pharmacokinetic investigations are being carried out, particularly when liver function is compromised or following hepatic transplantation. Conversely, since nephrotoxicity occurs with some of the metabolites, measurement of all the immunologically related products is likely to be more relevant in this clinical setting.

Radioimmunoassay (RIA) has been widely developed for use with serum or whole blood. The correlation between these two methods is good, although with RIA values are 30% to 70% higher, attributable to the presence of cross-reacting metabolites. Given these circumstances, it is important to specify the technique used for dosage recommendation based on derived pharmacokinetic values. Furthermore, since a number of the metabolites have some immunosuppressive properties, RIA may also provide a better index of total functional activity for any given amount of ciclosporine administered. With this assay, therapeutic levels for renal transplantation were found to range between 200 and 800 ng/mL and for the prevention of GVT in bone marrow transplantation were above 250 ng/mL. Meticulous observation of renal function and appropriate dosage adjustment is needed should creatinine levels rise or clearance rates fall; flexible titration in dosage is preferable to fixed regimens.

Trough levels measured by RIA prior to the next dose correlate with serum creatinine, episodes of renal rejection, nephrotoxicity, seizures and hepatotoxicity in renal and bone marrow grafting.

Some of the earlier assays employed polyclonal antibodies, whereas the availability of a mouse monoclonal protein raised against the parent compound gives values which are comparable to HPLC in the limits of detection and sensitivity, with a correlation of variation equal to or less than 7% in a similar sample volume.

A point of practical importance is the recognition that ciclosporine reversibly binds to plastic material, such as indwelling catheters, and spurious increases in drug level may result when samples are taken from the same line through which the drug was administered. A different lumen or direct venepuncture may be important.

MECHANISM OF ACTION – IMMUNOLOGIC

The central molecular event in ciclosporine-mediated immunosuppression is inhibition of interleukin-2 (IL-2) production by the CD4 positive subset of T-lymphocytes, and this occurs in a dose-dependent manner. Additional actions are attributable to an effect on macrophages and T-independent B-cell functions. We have shown in vitro (Jacobs; unpublished) that ciclosporine significantly inhibits the incorporation of tritiated thymidine into T-enriched mononuclear cell populations.
toxicity, when cholesterol concentrations are reduced to below 3.1 mmol/L.\(^{17}\)

**METABOLISM**

Metabolism occurs mainly in the liver via the inducible cytochrome P-450 system, with prominent biliary excretion of both residual parent substance and degradation products. However, enterohepatic circulation is limited\(^{15}\) and less than 10% of the metabolites appear in the urine. During catabolism, structural modifications consist of mono- and di-hydroxylated derivatives, N-demethylation and intramolecular cyclization,\(^{18}\) although the primary analogues excreted are the monohydroxylated derivatives M17, M1 and the N-demethylated product M21.

In humans, ciclosporine is the main component found in plasma, amounting to approximately 50% of the total area under the concentration time curve.\(^{16}\) However, due to a preferential distribution of this product and its metabolites, whole blood levels are generally higher than plasma concentrations, but are accounted for by only 27% of the parent substance.\(^{19}\) It is now evident that an enteroocyte first-pass phenomenon exists, probably also via P-450 catabolism, and this may further account for individual variability or fluctuations that reflect bowel damage or altered function.\(^{20}\)

**DRUG INTERACTION**

Many drugs will influence the metabolism of ciclosporine through their action on the cytochrome system, notably, cimetidine, ketoconazole, erythromycin, the oral contraceptives, androgens, methylprednisolone and some calcium channel blockers, all of which increase blood levels by competition for common metabolic pathways. Conversely, certain antibiotics, such as rifampicin, phenobarbital, phenytoin, carbamazepine and valproate, induce the cytochrome system, thereby decreasing the effective levels of this immunosuppressive agent.

**ELIMINATION**

Plasma clearance is tri-exponential, with half lives of 0.1, 1.1 and 16 hours\(^{15}\) and, in the steady state, systemic clearance is 6.5 mL/min/kg, with substantial inter-individual variation.\(^{21}\) This value is inversely related to the volume of distribution and age, being most significant under 10 years,\(^{22}\) with a 40% greater clearance, and children therefore require more frequent and higher doses. A similar finding is seen in bone marrow transplant recipients in comparison to solid organ grafts, where a lesser amount of drug is needed, with the difference being explicable on lower haematocrits in the former group, since red cells provide important ligands for ciclosporine binding.\(^{22}\) Conversely, clearance is slower in the elderly and in patients with decreased lipoprotein concentration and liver dysfunction.\(^{23}\)

**THERAPEUTIC MONITORING**

Although ciclosporine is an effective immunosuppressive agent, it has significant
toxicity, which can be reduced by careful monitoring. The recommended practice is to adjust dosage based on whole blood levels to compensate for variable absorption from the gastrointestinal tract. This approach does not take into account the relative role of the parent substance as opposed to metabolites and represents a compromise since it is not known how best to directly measure the total suppressive effects on the immune system.

High performance liquid chromatography (HPLC) is a well established technique which measures individually the parent substance and the various metabolites. This can be automated so that many samples can be determined with accuracy and precision, although the cost is high.24 This method may have advantages where specific pharmacokinetic investigations are being carried out, particularly when liver function is compromised or following hepatic transplantation. Conversely, since nephrotoxicity occurs with some of the metabolites, measurement of all the immunologically related products is likely to be more relevant in this clinical setting.25

Radioimmunoassay (RIA) has been widely developed for use with serum or whole blood.26,27 The correlation between these two methods is good, although with RIA values are 30% to 70% higher, attributable to the presence of cross-reacting metabolites. Given these circumstances, it is important to specify the technique used for dosage recommendation based on derived pharmacokinetic values. Furthermore, since a number of the metabolites have some immunosuppressive properties, RIA may also provide a better index of total functional activity for any given amount of ciclosporine administered. With this assay, therapeutic levels for renal transplantation were found to range between 200 and 800 ng/mL and for the prevention of GVHD in bone marrow transplantation were above 250 ng/mL.28 Metabolic observation of renal function and appropriate dosage adjustment is needed should creatinine levels rise or clearance rates fall; flexible titration in dosage is preferable to fixed regimens.

Trough levels measured by RIA prior to the next dose correlate with serum creatinine, episodes of renal rejection, nephrotoxicity, seizures and hepatotoxicity in renal and bone marrow grafting.29,30

Some of the earlier assays26,27 employed polyclonal antibodies, whereas the availability of a mouse monoclonal protein raised against the parent compound gives values which are comparable to HPLC in the limits of detection and sensitivity, with a correlation of variation equal to or less than 7% in a similar sample volume.31,32

A point of practical importance is the recognition that ciclosporine reversibly binds to plastic material, such as indwelling catheters, and spurious increases in drug level may result when samples are taken from the same line through which the drug was administered. A different lumen or direct venepuncture may be important.33,34

MECHANISM OF ACTION – IMMUNOLOGIC

The central molecular event in ciclosporine-mediated immunosuppression is inhibition of interleukin-2 (IL-2) production by the CD4 positive subset of T-lymphocytes, and this occurs in a dose-dependent manner. Additional actions are attributable to an effect on macrophages35 and T-independent B-cell functions.36 We have shown in vitro (Jacobs; unpublished) that ciclosporine significantly inhibits the incorporation of tritiated thymidine into T-enriched mononuclear cell populations
stimulated by mitogens and is efficient in the mixed lymphocyte reaction at concentrations as low as 100 ng/mL; this is a level that falls within the clinically therapeutic range. Furthermore, in our studies, this function was shown to be dependent on the presence of the agent from the beginning of the culture and at adequate concentrations, whereas its later addition had virtually no effect on DNA synthesis. The reversible impairment of IL-2 production extends to other lymphokines, including gamma interferon and interleukin-4 due to down-regulation of m-RNA transcription within the CD4 lymphocyte population. Interestingly, there does not appear to be concurrent inhibition of clonal proliferation in response to exogenous IL-2 by cells expressing receptors for this specific cytokine. Ciclosporine acts on cytotoxic T-lymphocytes indirectly by decreasing the IL-2-dependent proliferative responses and may also inhibit the precursor cytotoxic cells from acquiring or, more likely, down-regulating receptors. It has been shown that suppressor T-cell activities are relatively resistant to its action and, therefore, while the final molecular or cellular pathway remains to be elucidated, it might reasonably be speculated that imbalance between the unresponsive helper and the unrestrained suppressor functions accounts for the unique immunosuppressive activity of this substance.

When these molecular events are examined in the intact cell, a clear sequence emerges in which ciclosporine appears to diffuse into the liquid crystalline phase through the cell membrane, with recognisable consequences at a number of different levels. Thus, in the cytosol, interaction occurs between two calcium-dependent enzymatic complexes in the form of calmodulin and cyclophilin. Both of these have the protein-kinase activity that is known to be involved in signal transduction to the nucleus, leading to DNA synthesis, that initiates cell division in resting lymphocytes. In vitro studies have defined binding with a higher affinity for cyclophilin at 10.9 KD, leading to inhibition of the associated transcriptional pathway shared by most lymphokine genes in a dose-dependent manner. Ciclosporine selectively and in a linear fashion inhibits incorporation of tritiated thymidine by the isolated lymphoid nuclei, but this phenomenon occurs independently of blood concentrations. This represents another site of action, leading to direct modulation of lymphokine gene regulators, with inhibition of transcription of early phase T-cell activation genes (IL2, IL3, IL4, IFNalpha, TNF, GM-CSF and c-myc oncogene) that are essential for lymphocyte growth and differentiation. In contrast, this agent appears to directly interrupt the cell cycle at the G1-S interface, although once genes have been activated, the ciclosporine has no effect on sequential events.

The combination of these various molecular processes results in this substance exerting a significant influence on the thymus by decreasing the number of thymocytes indirectly through inhibition of lymphokine-induced maturation and directly by arresting the maturation of cells expressing the α and β T-cell receptors. The disruption of self-recognition facilitates the survival of precursors for autoreactive clones and possibly the acceptance of foreign grafts; withdrawal of the agent may then precipitate T-cell mediated autoimmune responses and so explain the development of GVHD after drug discontinuation in patients undergoing allogeneic bone marrow transplantation. Ciclosporine does not inhibit monocyte phagocytic functions directly, either in vitro or in vivo, but markedly disrupts the lymphokine-dependent T-cell lymphocyte macrophage interaction for the expansion of the immune response. At higher doses,
antigen presentation by macrophages seems to be directly affected, probably through associated prostaglandin E2 (PGE2) release.\textsuperscript{46}

**MECHANISMS OF ACTION – NON-IMMUNOLOGIC**

In a patient with lymphoblastic leukaemia (ALL), the concomitant administration of etoposide and cyclosporine resulted in the eradication of a previously refractory malignant infiltration of the bone marrow.\textsuperscript{47} In vitro studies seem to support at least two mechanisms for such a reversal of resistance to cytotoxics. In a pleotropic drug resistant subline of T-ALL, cyclosporine reversed the primary refractoriness to vincristine and daunorubicin, but had little activity in the drug sensitive paternal line. This action was unrelated to changes in the daunorubicin accumulation.\textsuperscript{48} Follow-up studies revealed that in a way similar to verapamil, cyclosporine restored the membrane potential of the resistant cells to those characteristic of the parent drug-sensitive tumour line.\textsuperscript{49}

In the multidrug resistant P388 cell line, this undecapeptide and some of its metabolites, in a dose-dependent fashion seems to increase the intracellular accumulation of the anthracycline, leading to a complete restoration of the chemotherapeutic response to this cytotoxic agent. The mechanism in this case seems to involve competition for an outward drug transport system that operates in the multidrug resistant cells.\textsuperscript{50} Although both verapamil and cyclosporine appear to be effective in overcoming drug resistance in malignant cells, evidence shows that cyclosporine\textsuperscript{46} is able to do this at clinically acceptable drug levels in the serum, with verapamil leading to unacceptable toxicity. This action appears to be compounded by a combination of these agents.\textsuperscript{50,51,52}

**CLINICAL STUDIES**

The in vitro observations have been applied increasingly to clinical practice and in each example animal experimentation has played a prominent role although, as experience has accumulated, some parallels as well as substantial differences between species have emerged.

**BONE MARROW TRANSPLANTATION**

These procedures have become firmly established as treatment options in aplastic anaemia, severe combined immunodeficiency, the acute and chronic leukaeamias and genetic disorders, including thalassaemia.

Three unresolved problems are, firstly, relapse with the haematologic malignancies, secondly, graft rejection despite careful matching and intense immunosuppressive conditioning regimens, and, thirdly, the emergence of reverse rejection or GVHD. In this latter situation, immunocompetent cells from the donor graft recognise antigenic disparity with the recipient and initiate firstly acute and then a chronic inflammatory response directed against target cells in the skin, mucosa of the gastrointestinal tract and biliary endothelium.\textsuperscript{53} Experiments in a mouse model showed that haematopoietic and lymphorcticular reconstitution followed infusion of
incompatible spleen cells and the emergence of GVHD was delayed after ciclosporine administration. In rats, this agent favoured the development of specific tolerance between donor and recipient. Our own studies in rabbits showed not only an improved survival, but a decrease in histologically proven GVHD in animals receiving this agent. Similar results have been reported by others. In extensive studies in dogs a high graft failure occurred among ciclosporine-treated animals, and although no deaths appeared to be directly related to GVHD, long-term tolerance was not induced. Subsequently, it was noted that methotrexate in combination with ciclosporine overcame the failure to engraft, with establishment of long-term effective haematopoiesis.

This experience with ciclosporine in allogeneic bone marrow transplantation is of interest, but none of the schedules reported under experimental circumstances uniformly abrogate GVHD, although incidence and severity appear to be favourably influenced.

Clinically it has been demonstrated that the cutaneous manifestations of established GVHD benefit in the short-term following ciclosporine administration. When this agent was given prophylactically for 14 to 48 weeks after grafting, a favourable outcome was documented, but in a proportion of patients GVHD followed its withdrawal.

In two prospective randomised trials conducted at the University of Washington comparing ciclosporine to methotrexate prophylaxis for GVHD in patients with acute nonlymphoblastic leukaemia (ANLL) in first remission and chronic myeloid leukaemia (CML) in the chronic phase, no significant difference was found regarding the incidence of acute GVHD or survival. However, patients receiving ciclosporine had a faster marrow engraftment, less mucositis and required fewer platelet transfusions. The disadvantages consisted of nephrotoxicity, tremors and hypertension. At follow-up between 3.2 and 6.2 years, both agents were seen to be comparable regarding the probability of survival, death from GVHD, incidence of interstitial pneumonitis, leukemic relapse and long-term survival.

Similar conclusions were reached by a Nordic controlled study regarding prevention of significant GVHD, although engraftment was faster, but in the subsequent report a significant increase in the leukaemic relapse rates in the ciclosporine group was found. Long-term survival and performance scores were similar in both groups.

A prospective Australian study showed comparable results, where relapse rates from leukaemia were found to be significantly higher in the ciclosporine group, and although survival was reduced, this was not significant. In a retrospective analysis involving 2036 recipients of HLA matched sibling bone marrow for acute leukaemia and aplastic anaemia, both ciclosporine and methotrexate were equally effective at preventing GVHD and superior to no prophylaxis. Sex disparity of female donors to male recipients was the most significant factor associated with acute GVHD.

In a further predictive analysis of risk factors for grade II–IV acute GVHD, the Seattle group demonstrated a reduction of this complication for every 100 ng/mL rise in the trough serum ciclosporine concentration of the previous week. The risk was 1.0, 0.6 and 0.2 for blood concentration of less than 100, 100 to 199, and 200 or more ng/mL of this immunosuppressive agent. However, due to the wide overlap in the ciclosporine serum levels, decreased readings had little predictive value for individual patients.

Acute GVHD proved to be the most significant factor for the development of the chronic variety of this phenomenon. The latter was also associated with a decreased
survival, and patients treated with either ciclosporine or methotrexate seemed to have a favourable outcome.

These studies have confirmed the value of ciclosporine as an effective agent in human bone marrow transplantation. Compared to methotrexate it had the advantages of faster engraftment and less mucositis, but was associated with frequent renal dysfunction, requiring blood level monitoring. The incidence and severity of acute GVHD had not been altered though, and there has emerged a rise in the leukemic relapses.

Following work on the canine model,60 the Seattle group conducted two randomised studies. In aplastic anaemia, standard methotrexate was less efficient than its combination with ciclosporine in preventing grade II–IV acute GVHD which did so without impairing sustained engraftment. However, chronic GVHD was increased when both drugs were used together. A possible explanation for this finding is that prevention of the initial mortality from the acute variant may have left a larger population at risk for development of this chronic complication.70-73 In ANLL,74 this combination was again superior to ciclosporine alone, with improvement in early survival and without a significant increase in the early relapse rate. Follow-up studies have shown that leukemic recurrence is adversely affected.73 Grade IV of this immune phenomenon was not observed in the combination group, whereas it arose in 29% when the agents were used singly.

Further analysis of patients transplanted for leukaemia showed that disease-free survival had been significantly better with the combination of methotrexate and ciclosporine in comparison to ciclosporine alone.73 Thus, when ciclosporine is combined with methotrexate for GVHD prophylaxis, three observations emerge. Firstly, the acute disease is reduced, with an improvement in the early mortality. Secondly, the chronic variant appears as a de novo phenomenon instead of evolving progressively from the acute changes. Thirdly, the latter developed after the withdrawal of the drug. It might therefore be of interest to extend the period of immunosuppression from 6 months to one year or longer, as is customary in solid organ transplantation.74 This was initially suggested by Baigalupo75 and recently reported by a Nordic group, confirming additional reduction in chronic GVHD.76

The inclusion of other agents to this combination has provided conflicting results. When prednisolone with ciclosporine was retrospectively compared to the same drugs individually or with additional methotrexate at a reduced dose, a protective effect was obtained against significant GVHD without obvious increase in the relapse rate.77 However, in a prospective study by the Seattle group this combination did not improve survival over the standard two-agent prophylaxis and, paradoxically, an increase in the incidence of GVHD was reported in the group receiving corticosteroid with infusion of the graft.78

Chronic GVHD, characterised by intrinsic immunosuppressive effects and the emergence of autoimmune phenomena, has been correlated with a protective effect from leukemic relapse, giving rise to the term graft-versus-leukaemia (GVL).79 This observation has been more clearly defined in animal models, but controversy persists as to whether the mechanisms for GVHD and GVL are identical. There are suggestions that when careful immunophenotypic studies are carried out, subtle differences in the composition of T-cell populations characterise the two immunologic phenomena.80

The ex vivo purging of lymphocytes from the bone marrow unequivocally reduces
the incidence of acute and chronic GVHD,\(^\text{81}\) and in a recent study use of the monoclonal antibody, Campath IgG 2b virtually abolishes this phenomenon.\(^\text{82}\) However, at least in some studies, an increase in leukaemic relapses have been reported,\(^\text{83}\) most strikingly in patients with chronic granulocytic leukaemia (CGL).\(^\text{84}\) There is the opinion that effective immunosuppression with ciclosporine-containing combinations may also lead to leukaemic relapse comparable to that associated with T-cell depletion (Personal communication: R Clift). It is nevertheless of interest that in CGL leukaemic recurrences form T-cell depleted grafts can be rescued by unfractionated second transplants without the need for intense preconditioning and when this is followed by standard ciclosporine and methotrexate prophylaxis for GVHD, substantial relapse-free survival is possible.\(^\text{85}\) This data and the evidence that even donor lymphocytes may reverse the relapse of a T-cell depleted graft point to GVL having a prominent effect in the control of the disease.\(^\text{86}\)

This issue is at present unresolved, and the challenge remains to determine precisely any distinction that may exist in the population of lymphocytes responsible for the two immunological syndromes. In the meantime, clinical trials in combination with appropriately designed laboratory studies should seek to clearly segregate the effects of ex vivo manipulation of grafts by means of monoclonal antibodies, either singly\(^\text{87}\) or as a cocktail. This should then be compared, in a controlled fashion, to other modalities, looking at the emergence of GVHD or GVL in matched groups of patients receiving unfractionated marrow grafts. Effective post-transplantation immunosuppression should be achieved with agents that on the one hand successfully abrogate the secondary disease and on the other retain the desired protective effects against leukaemic relapse.

SYNGENEIC GRAFT-VERSUS-HOST DISEASE

The conditions required for GVHD reactivity were postulated 20 years ago by Billingham\(^\text{88}\) and included three essential criteria: genetically determined histocompatibility differences between donor and recipient, immunocompetent cells in the graft capable of mounting an immune reaction against foreign histocompatibility antigens of the host and the inability of the host to recognise and initiate an immune response against the graft. GVHD has been reported after identical twin transplantations\(^\text{88}\), but this reactivity was limited to three weeks only, with resolution of all manifestation by 28 days. A similar condition has also been described following autologous bone marrow transplantation in 8% of cases.\(^\text{89}\)

Animal studies demonstrated that GVHD can be consistently induced after syngeneic grafting following lethal irradiation and withdrawal of ciclosporine. This phenomenon would start after a week, with typical manifestation in the skin, gut and liver.\(^\text{90}\) There was a rapid progression to the chronic variety within a week, with the usual histologic features.

Immune phenotyping studies in the acute variety revealed infiltration of target tissues with immature thymocytes. In the chronic form, both immature and mature T-cells were present.\(^\text{91}\) Since syngeneic GVHD seemed to confer a protective effect from malignant recurrence, as determined in an animal model,\(^\text{92}\) a pilot study was undertaken to deliberately induce this syndrome in a group of subjects undergoing marrow transplantation for resistant malignancies.\(^\text{93}\) Although all four developed this complication, only two required immunosuppressive therapy. It remains to be
determined if a corresponding effect can also be directed at solid tumours and, if so, can it then be applied to individuals with poor prognosis malignancies?  

APLASTIC ANAEMIA

Soon after the initial trials of bone marrow transplantation for aplastic anaemia were performed it was observed that when patients were conditioned with cyclophosphamide a proportion of individuals rejected the infused marrow and autologous recovery of the haemopoiesis would follow. Based on the observation in vitro that in aplasia immune mechanisms may suppress haematopoiesis, a number of studies demonstrated the effectiveness of high-dose methylprednisolone, antilymphocyte globulin and ciclosporine. However, since monoclonal antibodies against T-cells have proven to be ineffective in this disease, the exact mechanism for the action of ciclosporine remains to be established. Antilymphocyte globulin is an impure protein and, in vitro, displays both suppressive and stimulatory activities. A theoretical interaction between these two opposing actions may explain the observed responses.

In two prospective trials comparing this agent to antilymphocyte globulin, both were found to be equally effective in the acute phase and useful as salvage therapy in those unresponsive to the immunoglobulin preparation. Responses seem to be more rapid if corticosteroids or other immunosuppressive drugs are employed in combination with ciclosporine, but in some this outcome will not be sustained. Patients with granulocyte counts below \(0.2 \times 10^9/L\) generally derive less benefit from this form of therapy and similarly for treatment with antilymphocyte globulin. A recent controlled study has suggested that the addition of ciclosporine to an antilymphocyte globulin and prednisone-containing regimen is most effective in this severe category. However, the outcome of patients in the ALG arm was less good when compared to data from other series, leaving unclarified the role of the fungal agent in these patients, particularly when doses of ALG exceed 100 mg/kg.

PURE RED CELL APLASIA (PRCA)

A variety of immunosuppressive manipulations have been shown to be effective in this disorder of the red cell precursors, including cytotoxic agents, splenectomy, corticosteroids, antilymphocyte globulin and high dose gammaglobulin in fusion.

Ciclosporine is also active in the primary variant, when used alone or in combination with corticosteroids; in a subgroup, maintenance therapy may, however, be necessary to sustain improvements in erythropoiesis. When this condition is associated with chronic lymphoid leukemia or the Diamond Blackfan syndrome, good responses were achieved, even when patients were insensitive to steroids. Due to the rarity of the disease, this collective experience is based on retrospective studies and case reports. As a result, no dependable recommendation can be made for its use in these patients.
OTHER IMMUNE HAEMATOLOGIC DISORDERS

Immunologically mediated cytopenias may be the result of disturbances in either cellular mechanisms or by the elaboration of specific antibodies, which may then be detected on cell surfaces and exemplified by neutrophil or antiplatelet antibodies.\(^{118}\) Thus, in thrombocytopenic purpura (ITP) the conventional treatment is the use of immunosuppression with corticosteroids. In treatment failures, splenectomy has a high salvage rate and in a small number of relapses cytotoxic drugs will be effective in approximately 60% of patients.\(^{119}\) Of interest are those individuals who remain symptomatic following the failure of conventional treatment, and here innovative approaches are appropriate. In several case reports\(^{120-122}\) ciclosporine has resulted in improving platelet counts, although long-term maintenance of therapeutic blood levels of the agent is needed. In this context, the high cost of ciclosporine needs to be balanced against the indications for therapy in these patients, many of whom are asymptomatic or with platelet counts that are not life-threatening. In those who have developed refractoriness to platelet transfusions by isoinmunisation, ciclosporine has been shown to decrease the antibody concentration on cell surface, related to changes in the CD4 to CD8 lymphocyte ratios,\(^{123}\) leading to improvement in transfused platelet counts.

Other anecdotal reports have suggested response in immune neutropenia, associated with large granular lymphocytes,\(^{124,125}\) in adult onset cyclic neutropenia,\(^{126}\) or in Felty’s syndrome.\(^{127}\) In all these situations the same caveats apply to the use of expensive and potentially toxic agents on the basis of minimal information and, in the absence of properly controlled prospective studies, routine use cannot be recommended. In haemophiliac patients who have acquired antibodies to factor VIII, particularly in those refractory to the combination of plasma exchange, corticosteroids and cytotoxic immunosuppression therapy, there are intriguing suggestions that ciclosporine may have a role to play,\(^{128}\) but here again the need for protracted maintenance therapy would suggest a more balanced approach.

TOXICITY

Acute and chronic toxicity has been documented in many animal models over a wide range of oral and parenteral administration schedules. Nephrotoxicity is by far the most frequent side effect and has been described in autoimmune disorders where kidneys are apparently quite normal.\(^{129}\) Of greater importance, however, are the effects observed when ciclosporine is administered to patients undergoing bone marrow transplantation after intense preconditioning. Here, animal studies\(^{130}\) and case reports support the observation that cyclophosphamide and total body irradiation can induce renal damage at the clinically employed doses.\(^{131,132}\) The histological findings included mesangiolysis, severe arteriolonecrosis, hyalinization, luminal proliferation and thrombosis, with these changes evolving over several weeks.

Further compounding the picture is the fact that febrile patients concurrently receive several nephrotoxic drugs, including aminoglycoside antibiotics and loop-acting diuretics, thus rendering incrimination of purely ciclosporine related renal dysfunction notoriously difficult. Acute toxicity is frequently associated with elevated ciclosporine serum levels, but in most instances biochemical tests of renal
dysfunction are reversible upon reduction of the dose or discontinuation of the treatment. On the other hand, and in our experience, renal function was significantly disturbed when this agent was given over prolonged periods of time, despite meticulous monitoring of blood levels.

Clinical experience shows that in normal individuals, within four weeks of initiating therapy, a proportion will develop elevations in serum creatinine concentration, body weight and systemic blood pressure together with functional alteration of the intrarenal haemodynamics.

Bolus injections of this agent to rats induce similar changes, associated with catecholamine release and increase in the vascular resistance, leading to the suggestion that continuous infusion over 4 to 24 hours reduces these side effects. Tubular dysfunction due to ciclosporine toxicity causes impairment in the secretion of urea, reduced excretion of sodium, potassium, phosphate, bicarbonate reabsorption and hyperchloremic acidosis. Hypomagnesemia and renal magnesium wasting have been found, with significant hypertension, and were unrelated to ciclosporine blood levels, but both this agent and corticosteroid administration have been implicated. Replacement of magnesium may be of significant value in the management of blood pressure raised under these circumstances.

Although drug levels may not be predictive for the individual patients developing GVHD or renal toxicity, levels above 250 ng/mL generally correlate with renal dysfunction. Patients having undergone grafting and reasessed after 6 months of ciclosporine immunosuppression showed abnormal renal functions but when again examined at 1 year, 6 months after stopping this treatment, this had reverted to pretreatment values. However, experience in solid organ transplantation indicates that 10% of those exposed to this agent for prolonged periods (±10 years) may nevertheless develop irreversible renal damage.

Renal impairment with red cell fragmentation frequently seems to be related to GVHD and ciclosporine prophylaxis, but infrequently with methotrexate. Features of disseminated intravascular coagulation have also been described and reversal has followed reduction of the ciclosporine dose. The microangiopathy may be severe, but the histologic picture differs from that of classic haemolytic uremic syndrome or thrombotic thrombocytopenic purpura.

Hepatotoxicity is characterised by rising bilirubin, and in our experience of individuals undergoing allogeneic bone marrow transplantation two points emerged. The altered biochemistry rarely occurred in the absence of prior disturbances in renal function and adjustment of ciclosporine dose led to a rapid reversal in the bilirubin levels. Studies in animals showed the histologic picture to be that of centrilobular fatty change, hepatocyte necrosis with numerous autophagic vacuoles, and a dilated endoplasmic reticulum. Chronic drug administration induced hepatic dysfunction associated with an increased incidence of cholelithiasis.

In a large proportion of patients a marked increase in the serum triglycerides levels was described, without obvious evidence of liver abnormalities. Interestingly, however, one case that became symptomatic with visual and neurologic abnormalities reversed only with emergency plasmapheresis.

Although ciclosporine is not believed to cross the intact blood brain barrier, neurologic side effects are common. The most frequent are tremors and painful acral paresthesias. Occasionally, convulsions and confusional status have been described. Rare serious complications such as motor, cord and cerebellar syndromes arise, but reverse after discontinuation of the drug. On CT scanning this neurotoxicity
CICLOSPORINE IN HAEMATOLOGY

seemed to be associated with enhanced water content in the brain\cite{9}, as demonstrated by densimetric measurements.

Miscellaneous side effects have been extensively described and include hypertrichosis, increased incidence of infection, gingival hyperplasia, anorexia, nausea and vomiting.\cite{1146}

CONCLUSIONS

Ciclosporine has been shown to be an effective immunosuppressive agent and research into its unique mechanism of action has helped to understand many of the intricate pathways in the immune response. In haematologic practice the main use is in bone marrow transplantation. Here, its benefits have been enhanced by combination with other agents, such as corticosteroids or methotrexate, particularly in reducing the severity of acute GVHD. However, in some circumstances this appears to be offset by increasing leukaemic relapses and it therefore remains to determine its role in comparison to alternative options, that include in vivo T-cell depletion and ex vivo manipulation of the graft by exposure to a growing range of monoclonal antibodies. In aplastic anaemia, survival approaches that achieved with antilymphocyte globulin and additionally may salvage antilymphocyte globulin resistant patients. However, the quality of haematopoiesis differs from that achieved with transplantation in that response rates with the immunomodulation are less stable and there is an increase in incidence of clonal diseases that includes paroxysmal nocturnal haemoglobinuria, myelodysplasia and leukaemia being reported.

Other clinical studies have been largely anecdotal and suggest that in a variety of blood dyscrasias thought to have an immunological component, some improvement is possible in response to ciclosporine administration. Currently, data from prospective controlled studies are unavailable and until these are carried out, the use of this agent in patients with immune thrombocytopenia or pure red cell aplasia will be largely determined by individual preference.

Similarly, in malignancies the concomitant prescription of ciclosporine with some of the cytotoxic agents suggests that acquired drug resistance may be decreased and trials using this approach in myeloma and acute leukaemia are needed to confirm its practicality.

Although ciclosporine is widely used, its attraction is offset by its unquestioned toxicity, even when blood levels are meticulously monitored. Therefore, the immediate issue to be resolved is the detailed mechanism by which it exerts these side effects so that the best balance can be achieved between safety and the desired immunosuppressive effects.

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Rationale and Influence of Cyclosporin A Donor Pretreatment

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I. INTRODUCTION

Allogeneic bone marrow transplantation is the preferred form of treatment for aplastic anemia (1), lethal immunodeficiency and storage diseases (2), as well as acute (3,4) and chronic granulocytic leukemia (5). The initial, and often less than critical, enthusiasm for this procedure has been tempered by recognizing that substantial morbidity and mortality occur, with much of this attributable to GVHD. Paradoxically, since this latter unique immunologic entity may be associated with a beneficial antileukemic effect (6), attempts are being made to define its direct role, segregate associated but distinct mononuclear cell mechanisms, and then to harness and direct the way in which the specific effect or mechanism can be employed. Selective removal or functional impairment of subpopulations within the lymphocyte and monocyte fraction contained in the graft may vary cytolytic destruction of the skin, gastrointestinal tract, and biliary endothelium, while concurrently favoring the emergence of tolerance and varying degrees of immunologic reconstitution.

The basis for understanding these problems resides in a sequence of events that was recognized and described more than 30 years ago as the graft-vs-host reaction (GVHR) (7-9), in which three essential components have been delineated. First, the host must be incapable of rejecting the foreign bone marrow; second, a degree of histoincompatibility must exist between donor and recipient; and third, the infused graft must be immunologically competent and contain T lymphocytes. There is evidence that the effects of T lymphocytes are mediated by cytokines (10-14), and conceivably GVHD can serve as a model for autoimmune diseases (15-17).

In the context of bone marrow transplantation, each of the three steps mediating GVHR is amenable to manipulation.

First, there is little difficulty in achieving a high percentage of graft acceptance. Generally transplant recipients are prepared by immunosuppressive chemotherapy alone
or in combination with irradiation. Cyclosporin A, especially if used in combination with methotrexate, may have a graft-facilitating effect (18).

Second, absolute histocompatibility between donor and recipient should theoretically preclude the development of GVHD. However, and apparently in defiance of classical transplantation dogmas, this entity may follow syngeneic (19) or autologous grafting (20). One explanation is that the conditioning regimens to which the recipient is exposed may bring about an imbalance between autoreactive lymphocytes and suppressor cells, and studies from humans and rodents support this concept (12). Among HLA-identical allogeneic transplant recipients, 30% to 50% develop GVHD (21).

Third, the composition of the graft can be modified to facilitate hematopoietic reconstitution and simultaneously to modulate the mononuclear cells mediating the immune response. This can be achieved in two different ways. Effector cells may be quantitatively impaired by ex vivo purging with a single monoclonal antibody, such as Cam- path-1 (22) or a cocktail (23), both of which may be directed against a relatively wide range of cells. Conversely, it may be more rational to select biological reagents that selectively remove the helper-inducer as opposed to the helper-suppressor subsets (24,25). However, there may be retarded immunologic recovery, leaving recipients susceptible to infection or recurrence of neoplastic disease (26). Accordingly, it is attractive to consider the alternative approach in which the entire spectrum of mononuclear cells in the graft is infused, but a qualitative or functional lesion is produced, the intensity of which can be titrated to damp down the emergence of GVHD, permitting the development of tolerance. One agent that can potentially function in this way is cyclosporin A. In bone marrow transplantation it has been employed with relatively limited success as the sole postgraft means of treating the recipient. Virtually no data are available on ex vivo incubation with the collected marrow. Alternatively, it can be used to pre-treat the donor and the recipient, with subsequent reduction in dose to achieve the desired effect in the latter.

The present studies were aimed at establishing the feasibility of the latter approach in preventing acute GVHD and, concurrently, to document any risk to the donor of short-term exposure to this agent.

II. CYCLOSPORIN A

Cyclosporin A was discovered in early 1972 in the Research Laboratories of Sandoz Ltd in Basel. It is a hydrophobic cyclic peptide consisting of 11 amino acids with a molecular weight of 1203. The active compound is a fungal metabolite derived from cultures of Cylindrocarpon lucidum or Trichoderma polysporum (27). This agent has recently been extensively reviewed (28,29).

Available evidence localizes the action of cyclosporin A to the inductive phase of response in the immunocompetent cells as they encounter antigenic challenge. Cell division, by way of contrast, does not appear to be affected and, furthermore, no lymphocytoxic effects are demonstrable. The reduction of lymphoblast numbers, which may be striking in vitro, probably reflects prevention of stimulation rather than elimination of blast cells by cytolytic mechanisms (30). Expressed in another way, the primary effect of this agent is directed against the earliest cellular events in the lymphocyte that follow mitogenic stimulation and finds expression by preventing transformation into the blast cell.

Cyclosporin A does not in all models lead to selective clonal deletion, but rather to the induction of a temporary suppressive state that is reversible when treatment is termi-
nated (31,32). The availability of monoclonal antibodies may help resolve this difficulty. For example, CD4\(^+\) T lymphocytes are now divisible into helper-suppressor, or CD4\(^+\)2H4\(^+\), in contrast to helper-inducer, or CD4\(^+\)4B4\(^+\), subpopulations (24,25).

III. EXPERIMENTAL STUDIES

A. Rabbits

New Zealand white (NZW) and R strain animals, having an average weight of 2 kg, were used throughout. They were individually housed and received an unrestricted supply of water, containing prophylactic sulphaquinoxaline sodium (Embasin, May and Baker, Dagenham, England), and a standard rabbit diet (Epol, Johannesburg) medicated with Amprolium (Merck and Company, Inc., USA). Temperature and weight were regularly charted. A full blood count and differential were carried out twice weekly. The biochemical profile was monitored regularly.

B. Radiation Technique

The rabbits were rendered aplastic by delivering 1200 cGy total body irradiation from a cobalt source placed 100 cm above the midplane of the prone but anesthetized animal. Radiation rates between 40 and 80 cGy/min were shown not to differ significantly. We have previously demonstrated (33) that uniform and irreversible aplasia is produced without unacceptable complications. Specifically, no gastrointestinal tract lesions were demonstrated. Furthermore, there was no difference between this technique and fractionating the radiotherapy, as suggested by others (34,35). In occasional animals unexplained death occurred within 24 hours after radiotherapy, ascribed to "radiation shock." In another series of experiments the alternative radiation dose rate at 8 cGy/min was examined, but this regimen was not found to be associated with less toxicity than the more rapid exposures.

In a series of controlled experiments, one or both of the femurs were shielded with lead, and in another group autologous reconstitution was undertaken to monitor both optimal rates of hematopoietic recovery and isolate possible complications of the radiotherapy and transplantation procedure itself.

C. Allogeneic Bone Marrow Transplantation

Thirty-six hours after completing lethal whole body irradiation, the animals underwent allogeneic bone marrow transplantation. Between 2 and 4 \(4 \times 10^8\) nucleated cells per kilogram were infused from R females into NZW males. Standard mixed lymphocyte cultures (36) to demonstrate nonidentity at the major histocompatibility complex were not uniformly successful. However, using a modified technique (37), antigenic disparity was demonstrated between the two strains; this was most striking when mitomycin-treated lymphocytes derived from the mesenteric lymph node were used as antigen and appendixal lymphocytes as responding or target cells. Results at day 3 gave stimulation indices between 3.5 and 13.9. Even the latter technique does not produce absolutely uniform results, and we have subsequently demonstrated that mixed lymphocyte reaction, with minor modifications, can reliably predict histoincompatibility (Jacobs and Paulsen, unpublished).
D. Cyclosporin A Administration

Rabbits were given 10 mg/kg daily of cyclosporin A by intramuscular injection for 28 days following transplantation. Plasma levels were monitored by high-pressure liquid chromatography.

E. Results

1. Radiation Controls

Irradiated animals developed increasing pancytopenia, with platelet and granulocyte counts reaching their nadir between days 4 and 6 (Fig. 1), and all the rabbits died. Median survival (Fig. 2) was 6.8 days (SE ± 0.08; range 2-11 days).

Autopsy studies at intervals showed rapidly decreasing bone marrow cellularity, and death was uniformly due to infection, with widespread colonies of bacteria present in all organs. Minimal associated hemorrhage was present in the gastrointestinal tract of occasional rabbits. There was profound loss of lymphoid tissue throughout the gut, thymus and spleen (38).

2. Femoral Shielding and Autograft Rescue

In animals having one or both femora shielded, reduction in white cell and platelet counts were comparable to those observed in radiation controls, but peripheral values returned rapidly to reach normal by day 21 (Fig. 1). The bone marrow, when studied serially, showed clonal regeneration of the previously aplastic medullary spaces, and histology was normal by day 30.

In the autografted animals who received $2 - 4 \times 10^8$ nucleated cells/kg, peripheral blood values were indistinguishable from those observed with femoral shielding (Fig. 1). Median survival (Fig. 2) was not reached at any time during this study, and only an occasional death occurred.

![Graph](image_url)

**Figure 1** Peripheral blood granulocyte and platelet count. The fall in circulating levels and their subsequent return to normal is not statistically different in the three groups of animals. Data are expressed as a percentage of mean basal values and plotted against elapsed time in days. (Reproduced with permission of the Editors and Publishers of Experimental Hematology Today 1981, S. Karger AG, Basel.)
Cyclosporin A Donor Pretreatment

![Graph showing survival rates](image)

**Figure 2** Observed survival in rabbit experimental studies. The median survival for irradiated controls was 6.8 days. Following allogeneic bone marrow transplantation without immunosuppressive agents, survival was 15 days and none were alive at 100 days, whereas in allografted animals receiving cyclosporin A, median survival was 40 days and 33% were alive and well at 100 days. The incidence of histologically proven GVHD in the allografted controls was 80% and in the cyclosporin A-treated animal this was below 25%. (Reproduced with permission of the Editors and Publishers of Experimental Hematology Today 1981, S. Karger AG, Basel.)

Anemia aggravated by repeated blood sampling was prevented by the use of microtechniques. In these animals, as opposed to radiation controls, daily oral prophylaxis with 10 ml trimethoprim combined with 50 mg sulphamethoxazole decreased deaths from infectious episodes and is routine practice in the animal transplantation program.

3. **Allogeneic Bone Marrow Transplantation**

In the allograft controls 2-4 × 10^8 nucleated cells/kg resulted in granulocyte and platelet reconstitution patterns in the peripheral blood that was comparable to that demonstrated by animals undergoing radiation with femoral shielding or after autografting (Fig. 1). The same findings were present with bone marrow on serial histologic studies.

Since the study was designed to examine the effect of cyclosporin A on the incidence and severity of GVHD, the rare animal dying before engraftment could be demonstrated was excluded from analysis.

The clinical findings in the allografted recipients were dramatically different from those in the autograft controls. Gross weight loss was the striking feature, and at day 40 respective values were 0.8 and 2.8 kg. There was, in addition, patchy but characteristic extensive hair loss. Diarrhea was a variable feature, with cultures generally being negative.

Survival of control animals (Fig. 2) was 10% at day 40 and 0% at day 100. The cause of death was typically infection in the respiratory tract, with nasal discharge of pus. Pneumonia was often present, with widespread destructive abscess formation being a feature. Histology of the skin, gastrointestinal tract, and liver were diagnostic of GVHD. In the skin, aggressor lymphocytes were present in the epidermis, and spongiosis was prominent, particularly at the dermoepidermal junction. In the gastrointestinal tract loss of
glands and patchy to total mucosal denudation were most striking in the small bowel. The liver showed infiltration of lymphocytes, loss of the limiting plate and destruction of bile ducts by lymphocytes. Generalized lymphoid atrophy was evident in the spleen, lymph nodes, and thymus. These features were similar to those reported both in man (39) and in experimental animals.

4. Cyclosporin A-Treated Recipients
Two striking features were evident in these animals. First, they were uniformly in superior clinical condition, with weights not significantly different from autograft control animals. Hair distribution was normal and diarrhea was not present. The second was the markedly different survival rate between allografted controls and rabbits receiving cyclosporin A after bone marrow transplantation (Fig. 2). At day 40 10% of the allograft controls were alive compared to 44% of those receiving cyclosporin A. This difference was even more obvious at 100 days, when none of the controls survived but 33% of those that had been treated were alive and well, with normally functioning donor grafts.

Of particular note was the fact that the histologically recognizable features of GVHD, which characterized the allografted control animals, were present in only 25% of animals receiving cyclosporin A. Furthermore, in the latter group death due to typical GVHD was unusual and was often attributable to an incidental cause, such as perforated gastric ulcer. However, infection did occur and was most commonly due to bronchopneumonia and septicemia; it is presently not clear whether the latter may be unusual expressions of GVHD modified by cyclosporin A therapy.

5. Cyclosporin A Pretreatment of Donors and Recipients
In experiments where donors were pretreated for 7 days with cyclosporin A and the recipients continued to receive this agent for 28 days after transplantation, the survival curves (not shown in Fig. 2) were not statistically different from those where only the recipients were treated. Furthermore, examination of the tissues in this group and cause of death were also similar to the cyclosporin A-treated recipients.

F. Comments
Three points are relevant. First, in the cyclosporin A-treated allograft recipients, the death rate appeared to accelerate after day 28 when this agent was discontinued. It, therefore, remains possible that longer periods of drug administration are necessary. These findings are entirely compatible with the suggestion that the action of this unique immunosuppressive agent is not to produce lasting tolerance produced by selective clonal deletion, but is a transient phenomenon related to the presence of critical concentrations in the body (31,32). Furthermore, there are interesting parallels between this observation and those reported in a human transplantation study (40,41).

Second, in contrast to experience reported with a similar model (Speck, personal communication, 1980) biochemical disturbances or other gross toxicity attributable to the cyclosporin A were not found.

Third, and perhaps in partial explanation for the lack of major side effects in our animals, was the absence of significant concentrations of this agent in the plasma when examined by high pressure liquid chromatography. It remains possible, therefore, that the dosage administered may have been inadequate or the batch used had less biological activity than we had anticipated. This query is the subject of further study. Nevertheless, it can be concluded that there is an unequivocal and marked reduction in acute
GVHD and that striking prolongation in survival can be achieved with plasma concentration of cyclosporin A that may be lower or less critical than published evidence would suggest. Indeed, it remains to be proven that plasma levels are the most valid reflection for the immunosuppressive properties of this agent and concentration in cellular compartments or control by functional assays, such as mitogen response, may be more appropriate. Nevertheless, the measurements are clearly the best means currently available for monitoring patient compliance and drug absorption, and, therefore, to anticipate the development of toxicity.

IV. CLINICAL STUDIES

Difficulties exist in the prevention (42,43) or abrogation of GVHD (44,45), and while cyclosporin A is beneficial in a number of transplant situations (46), including marrow grafting (41,47), it is less than totally effective and appears approximately equivalent to methotrexate (48). It does not appear that the incidence or severity of the acute syndrome, with its high morbidity and mortality, or that of the chronic variant, with its relentless progression and mutilating side effects, can be eradicated with this agent alone. These observations raise the question that at least the acute disease might reflect transfer of immunocompetent lymphocytes with the graft and that such a situation might, theoretically at least, be amenable to donor pretreatment. In view of the limited side effects observed in animals and while recognizing that any responses in man may be different, it seemed appropriate to examine additional donor pretreatment with cyclosporin A in a clinical bone marrow transplantation program.

A. Materials and Methods

Fifty-one patients underwent allografting on a program approved by the Ethics and Research Committee of the University of Cape Town and Groote Schuur Hospital. Donors and recipients were fully informed and their participation required signed consent.

Initially, patients with refractory acute leukemia were offered the procedure but subsequently only those in consolidated complete remission or having severe acute aplastic anemia were considered eligible. Sibling donors compatible at the major histocompatibility complex and nonreactive with their recipients in the mixed lymphocyte culture were used.

Severe acute aplastic anemia was defined according to internationally accepted criteria (49) and patients were conditioned with 50 mg/kg of cyclophosphamide by intravenous injection on four consecutive days before transplantation (50). The patients with acute leukemia were treated with a combination of stoposide, cytosine arabinoside, and doxorubicin (51), after which they were conditioned with cyclophosphamide, 60 mg/kg on 2 consecutive days, and 1000 rads total body irradiation at a maximum exposure rate of 7 rad/min given 24 hours after the last dose of conditioning drug. All patients were managed in reverse isolation, and received hyperalimentation and such antibiotic and transfusion support as was appropriate.

Three different immunosuppressive regimens were evaluated in the course of this study (52). In group 1 (n = 23) cyclosporin A was not available, and following transplantation patients received only methotrexate (50). In group 2 (n = 8) cyclosporin A was commenced the day before infusion of the graft at an oral dose of 12.5 mg/kg twice a day for a week and then 6.25 mg/kg twice daily for the next week, after which adjustments
were made to maintain plasma concentrations between 250 and 500 ng/mL by radioim-
monoassay (53) at least once a week for the entire duration that patients received this
immunosuppressive drug. In group 3 (n = 20) both donor and recipient commenced
cyclosporin A on the same dosage schedule 2 weeks before transplantation; patients re-
mained on this agent for the same duration as those in group 2. At all times, plasma con-
centrations were monitored and doses adjusted at least once every week to maintain levels
in the same therapeutic range as in group 2.

Biochemical measurements of renal and hepatic function, as well as electrolyte
status and acid-base balance, were measured on alternate days in donors and recipients.
Any increase in creatinine or urea values, even while still in the normal range, was man-
aged by small doses of oral furosemide, and failure to immediately reverse this trend re-
quired reduction in cyclosporin A dosage. In the donors, as well as in the recipients,
these measurements were continued on an outpatient basis.

Acute (50) and chronic (54) GVHD were defined according to established criteria.
Both syndromes were treated by continued administration of cyclosporin A; in the acute
disease methylprednisolone was added; in the chronic form additional high-dose pulsed
methylprednisolone (45) was initially used, followed by long-term corticosteroids and
azathioprine. The dose of the two drugs was variable and started at 0.5 mg/kg and 2 mg/
kg of lean body mass, respectively. Methylprednisolone was gradually reduced, whereas
the dose of azathioprine remained constant; the efficacy of this two-drug combination
on the clinical course of the chronic GVHD was constantly reviewed; average duration
was generally in excess of 9 months.

B. Results

1. Methotrexate-Treated Patients

As shown in Table 1, these patients (group 1; n = 23) were treated with methotrexate for
immunosuppression (50). Ten of the 23 patients (43%) developed classical severe acute
GVHD within 6 weeks of transplantation, which was refractory to therapy and caused

<table>
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<th>Group</th>
<th>Acute</th>
<th>Acute, progressing to chronic</th>
<th>De novo chronic</th>
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<tr>
<td>1 (n = 23)</td>
<td>10</td>
<td>3</td>
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<tr>
<td>2 (n = 8)</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>3 (n = 20)</td>
<td>6</td>
<td>0</td>
<td>2</td>
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</tbody>
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Group 1: posttransplant methotrexate only. Group 2: posttransplant cyclosporin A only. Group 3:
donor and recipient cyclosporin A pretreatment and this agent continued after grafting in the recipient.

The incidence of acute GVHD was 43, 25, and 30%, respectively; p > 0.05.

Source: Reproduced with permission of the Editors and Publishers of the Scandinavian Journal of
Haematology, Munksgaard International Publishers Ltd., Copenhagen, Denmark.
death in six patients. Of the remaining four patients one died of Budd-Chiari syndrome, which may have been related to the long period of prior intensive chemotherapy, and three progressed to chronic GVHD, of whom one is alive with disfiguring cutaneous lesions, one died from disseminated tuberculosis without evidence of GVHD, and the other died following a seizure but with severe skin lesions of GVHD; autopsy failed to reveal the cause of death.

Chronic GVHD arose de novo, that is, without any prior acute disease, in two patients (9%) and has been self-limiting in both.

2. Cyclosporin A-Treated Patients
These patients (group 2; n = 8) received cyclosporin A immunosuppression without donor pretreatment. Severe acute GVHD occurred in two patients (25%) and in both responded promptly to additional corticosteroids. One patient died from leukemic relapse and the second from a ruptured cerebral aneurysm.

Chronic GVHD without an antecedent acute episode occurred in three patients (38%). One died from disseminated fungal infection with poorly controlled cutaneous grade I GVHD. The other two developed minimal but characteristic skin lesions 3 months after discontinuing cyclosporin A. Both have responded to restarting cyclosporin A in conjunction with a single course of pulsed high-dose methylprednisolone.

3. Cyclosporin A-Treated Patients and Donors
These patients (group 3; n = 20) and their donors were pretreated with cyclosporin A for 2 weeks before transplantation. Six patients developed early GVHD (30%). In two this was mild and responded promptly and completely to corticosteroid therapy; one patient subsequently died from massive gastrointestinal tract hemorrhage and the second patient is currently well. In the other four patients the disease was also mild, being defined as grade I cutaneous changes, diarrhea less than 500 mL in 24 hours and without abnormality on liver biopsy. However, in none of these patients could this clinical syndrome be reversed on therapy and all died within 3 months—three from infection and one from massive hemorrhage from a duodenal ulcer.

4. Engraftment
Engraftment is defined as the time taken from transplantation to reach granulocyte counts of 0.5 × 10^9/L or a platelet count of 25 × 10^9/L. These findings were substantiated by a bone marrow aspiration and trephine biopsy, which showed greater than 15% of hematopoietic cell repopulation. The median time to achieve these criteria for the three groups was 21 days (range 9-26), 14 days (range 6-33), and 13 days (range 7-16), respectively.

Actuarially predicted survival curves (Fig. 3) show little difference for median survival, with a nonsignificantly higher plateau for patients in group 3.

5. Toxicity
In none of the donors did biochemical tests of renal function rise outside the normal range during the 2-week period of cyclosporin A administration. The donors were all fully reassessed, including biochemical measurements, before discharge and again within 1 month after discontinuing cyclosporin A therapy. Furthermore, in a separate series of individuals with severe acute aplastic anemia without a transplant option (55), cyclosporin A was used as the primary form of immunosuppression either singly or in combination with antilymphocyte globulin; in 12 individuals monthly follow-up for an average period
Figure 3  Survival of bone marrow transplantation patients. Actuarially predicted survival curves showed no significant difference between patients receiving methotrexate alone (group 1; n = 23), those in which recipients only received cyclosporin A (group 2; n = 8), and where donors and recipients received cyclosporin A pretreatment and the recipients, in the post transplant period, continued with the same agent (group 3; n = 20). (Reproduced with permission of the Editors and Publishers of The Scandinavian Journal of Haematology, Munksgaard International Publishers Ltd, Copenhagen, Denmark.)

in excess of 9 months showed no late disturbances in biochemical tests of renal or hepatic function.

In recipients receiving cyclosporin A, transient elevation of blood urea and creatinine was seen in 50% of the patients in groups 2 and 3. Any increment in these biochemical measurements, despite their remaining within the normal range, was managed by treating the patient with 20-40 mg furosemide daily or twice a day. Failure to immediately reverse the trend required reduction in the dose of cyclosporin A being given. No examples of progressive deterioration or irreversible dysfunction were observed using this approach.

C. Comments

From these preliminary studies only three observations can be made. First, these overall results from bone marrow transplantation are less satisfactory than reported by others and those currently being achieved in our own program. This reflects the referral of patients during the early years of this program at a time when their clinical condition was poor, and, therefore, at least in those with aplastic anaemia, extensive prior transfusions may have further compromised the outcome.

Second, and based on these results, it appears as though the administration of cyclosporin A to the recipients alone reduced the incidence of acute GVHD and improved the response to its management when additional methylprednisolone was required.

Third, the additional pretreatment of the donor, albeit the number of patients transplanted was small, had the further benefit of reducing the severity of clinical manifestations.
In both of the latter two groups, and particularly where donors were pretreated, it is noteworthy that meticulous monitoring of plasma levels and prompt management of changing hepatic biochemistry made it possible to use this agent without unacceptable morbidity.

Finally, in the clinical context, no beneficial effect from either cyclosporin A regimens could be demonstrated on de novo chronic GVHD.

V. SUMMARY AND CONCLUSIONS

The use of bone marrow transplantation in clinical practice is rapidly increasing. It is the preferred method of treatment in some instances and in others forms an integral part of current management programs, particularly in hematologic malignancies. A major deterrent to allografting is the morbidity and mortality associated with acute and chronic GVHD. The approach to this problem is not uniform. In patients with aplastic anemia, the objective is to eliminate this entity completely. Conversely, and perhaps paradoxically, in patients with leukemia there may be a need to retain a controlled level of GVHD in order to harness an associated and beneficial immunologic effect on preventing relapse (56). If this is to be achieved successfully, then a greater understanding of the various components of acute and chronic GVHD are needed so that these can be manipulated to selectively enhance those that lead to immunologic destruction of residual tumor cells and suppress cytotoxic processes that cause unwanted destruction of normal tissue. However, there remains controversy as to the relative contributions from cellular populations that directly mediate one or other events, in contrast to humoral mechanisms that reflect monocyte or lymphocyte activation. Indeed, it is not only possible but likely that both arms of the immune response mechanism may be acting in concert.

There are a number of new and novel approaches currently being examined that include the exposure of the graft to ultraviolet irradiation (57,58), selective depletion of a wide variety of cells with monoclonal antibodies having differing specificities, or variation in the posttransplant immunosuppressive regimens. Each of these have met with differing degrees of success, but none have uniformly led to the control of GVHD. In the quest for alternative regimens with less cytotoxic effects, cyclosporin A has been extensively studied. Although the induction of a qualitative lesion in T-helper lymphocytes was demonstrated in vitro and while initial in vivo studies were encouraging, frustratingly, much of the early promise has not been fulfilled. On the theoretical basis that the acute syndrome may be amenable to better control with longer periods of effector cell exposure to this unique biologic agent, donor pretreatment followed by the administration of cyclosporin A to the recipient was examined in both an experimental rabbit model and a clinical bone marrow transplantation program. The results of the former studies showed no clear-cut benefit over its administration to the recipient alone. In contrast, clinical studies, while limited by the historical nature of the trial design and the small number of patients entered, provided some evidence for a decrease in the incidence and severity with donor pretreatment which could be achieved without demonstrable morbidity.

It might reasonably be concluded, at a time when all possible means are being explored for the control of GVHD (59), that this schedule justifies further study. It can be hypothesized that manipulation of exposure time and titration of dosage would lend flexibility to modulating the acute and chronic forms of this unique phenomenon. Thus, additional donor pretreatment may have a role in retaining the beneficial antileukemic
effects whilst concurrently preventing untoward damage to other organ systems in the recipient. Furthermore, with programs being rapidly expanded to include the use of mismatched siblings or phenotypically identical but unrelated donors, it also offers a wider choice in suppressing some of the very early events in the acute syndrome.

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Graft-vs.-Host Disease
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ORIGINAL ARTICLE

Assessment of converting from intravenous to oral administration of cyclosporin A in pediatric allogeneic hematopoietic stem cell transplant recipients

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We studied the administration method during a transition period from continuous intravenous (i.v.) infusion to oral administration of cyclosporin A (CsA). Thirty-two pediatric hematopoietic stem cell transplant (HSCT) recipients, between the ages of 8 months and 15.6 years (median 7.1 years) participated in this study. The pharmacokinetic properties of CsA was evaluated during the transition period from i.v. to oral CsA. The daily oral dose of CsA was three times higher than the i.v. dose. Oral dosing began immediately after the continuous infusion was discontinued. Whole-blood CsA concentrations were measured by a monoclonal fluorescence polarization immunoassay (FPIA). The mean ± s.d. value of bioavailability (F), maximum concentration (Cmax), half-life (t1/2) of CsA were 43.1 ± 14.4%, 1135.3 ± 340.6 ng/ml and 3.1 ± 1.2 h, respectively. Mean clearance (CL) ± s.d. was 480.9 ± 103.7, 414.9 ± 137.1 and 320 ± 51.8 ml/h/kg in patients <20, 20–40 and >40 kg of body weight, respectively. The CsA CL of younger children was significantly greater than for older children (P = 0.044). CsA trough levels were maintained within the therapeutic range throughout the transition period. Therefore, our findings suggest that the immediate administration of an oral formulation, after discontinuation of the continuous infusion, would be practical and effective for routine clinical use.

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Keywords: pharmacokinetic; cyclosporin A; hematopoietic stem cell transplantation; pediatric

Introduction

Among the variety of immunosuppressants available, cyclosporin A (CsA) has been the most extensively used drug to prevent or treat graft-versus-host disease (GVHD) in hematopoietic stem cell transplant (HSCT) recipients, since its introduction in the 1970s.1–6 It has been shown that CsA is effective in preventing and controlling acute and chronic GVHD in pediatric patients.7–8 However, the use of CsA in the clinical setting is complicated by significant intrapatient and interpatient variability in its pharmacokinetics.9,10 Prior reports have shown that there are several factors contributing to this variability, such as age, genetics, the physical condition of patients, type of transplant, post transplant time and concomitant medications.11 As was previously observed, the major cause for variability is the erratic absorption of CsA.12 Particularly, HSCT recipients have considerable difference in their gastrointestinal (GI) integrity, as compared to solid organ transplant recipients; these differences are in part caused by GI inflammation that develops after conditioning chemotherapy and radiation treatments and/or intestinal GVHD and viral infection.13–15 In addition, a significant factor for the interindividual variability is the patient’s intrinsic capacity to metabolize CsA.12 In general, many drugs administered to children are characterized by a substantially faster elimination than in adults; however, there are limited data on the pharmacokinetics of CsA in pediatric transplant recipients.16,17 Some studies have evaluated the pharmacokinetic properties of CsA in children undergoing organ transplantation.18,27 However, few published data are available for the pediatric HSCT population.28

Despite the widespread use of CsA, the administration method, dosage schedule and optimal dose of CsA have not been well established, and a variety of treatment protocols have been accepted at numerous transplantation centers.28 Several studies have suggested that the continuous intravenous (i.v.) infusion of CsA has been effective in preventing acute GVHD in HSCT recipients.29,30 Moreover, a continuous infusion is associated with a decrease in the incidence of serious CsA-related toxicities compared
with a short i.v. infusion. Based on these data, we administered CsA for a continuous i.v. infusion in the early period after stem cell transplantation; it was continued until the patient could tolerate oral dosing after recovery from treatment-related GI complications. The oral formulation was administered immediately after discontinuation of the continuous infusion with an attempt to maintain CsA levels and provide a practical management option.

The objectives of this study were to study the transition period between continuous i.v. infusion and oral CsA treatment by evaluating the pharmacokinetic properties of CsA in pediatric HSCT recipients.

Patients and methods

Patients

Children who received allogeneic HSCT and CsA for prophylaxis of GVHD at Samsung Medical Center were enrolled in this study. They had normal hepatic function (<1.2 mg/dl), SGOT and SGPT <3 × upper reference limit) and normal renal function (serum creatinine <1.5 mg/dl, infant criteria <0.6 mg/dl). All patients were treated with a study protocol that had been approved by the Institutional Review Board at Samsung Medical Center. Written informed consent was obtained from parents or guardians before the patients were allowed to enter the study.

Drug administration

Intravenous CsA (Cipol In®, 50 mg/ml, CKD, Korea) was started the day before stem cell infusion at a dosage of 5 mg/kg per day as a loading dose; it was given as a continuous infusion through an indwelling catheter. CsA injection was continued, at 3 mg/kg per day, from the day of stem cell infusion and then the dose was titrated to a level of 250–350 mg/ml. Thereafter, the oral formulation was substituted for the i.v. formulation when the patient was able to tolerate oral medications, after they recovered from treatment-related GI complications. The daily oral dose was administered immediately after discontinuation of the continuous infusion, three times the i.v. dose, in two equally divided doses, at 12 h intervals. Soft gelatin capsules (Cipol N®, 25 100 mg/cap, CKD, Korea) or an oral solution (Neoral® 100 mg/ml, Novartis) were administered, depending on their preference and developmental capability. All patients received anti-fungal prophylaxis with fluconazole, pneumocystis carinii prophylaxis with cotrimoxazole and anti-viral prophylaxis with acyclovir.

Blood sampling

Blood samples were collected during the transition period from i.v. to oral dosing. Whole blood (2 ml) was collected into tubes, with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Blood was taken 24 h before the transition, simultaneously from both the peripheral veins and the indwelling catheter lines, at 0 (just before transition), 1, 2, 3, 5, 7, 12 and 24 h after transition via the peripheral lines. As CsA can bind to the plastic tubing of a central line, blood samples were obtained from another lumen that was not involved in the infusion. The first 2 ml of blood was discarded when specimens were drawn from both peripheral veins and indwelling catheter lines. All blood samples were refrigerated until analysis. The results were determined within 48 h after sampling.

Analytical method of cyclosporin A concentration

Cyclosporin A concentrations, in whole blood, were measured at the biochemistry laboratory in this center by monoclonal fluorescence polarization immunoassay (FPIA) using commercially available assay kits (TDx, Abbott Laboratories, USA). The operating range for this method is 0–1500 ng/ml but the lower limit of detection is 25 ng/ml. The precision of this method was evaluated using three control concentrations of 150, 400 and 800 ng/ml. The within-run coefficients of variation were 1.3, 1.2 and 1.3%, respectively. The within-run coefficient of variation was estimated to be less than 2.5%. The analytic method used in this study, monoclonal FPIA, showed cross-reactivity with some CsA metabolites; however, it was chosen because the results are practically applicable in the clinical setting, and they provide good reproducibility.

Pharmacokinetic analysis

Pharmacokinetic modeling and parameter estimates were performed using WinNonlin version 3.1 (Pharsight Corporation, USA). Utilizing the raw data, such parameters as volume of distribution (Vd, ml/kg), absorption rate constant (Ka, h⁻¹), lag time (Tlag, h), bioavailability (F, %) and clearance (CL, ml/h/kg) were estimated from the pharmacokinetic model. Then, the elimination rate constant (Kel, h⁻¹) and elimination half-life (t1/2h) were derived from the primary parameters using the following formulae, respectively: Ke = CL/Vd, t1/2h = 0.693/Kel. The peak concentration (Cmax) and the time to reach maximum concentration (Tmax) were accepted as the observed values.

Statistical analysis

Comparisons of the pharmacokinetic parameters between two groups according to CsA formulations were performed using the two-sided Wilcoxon’s rank sum test. For three groups according to patient body weight, the Kruskal-Wallis test was used. Wilcoxon’s signed rank test was used to compare the difference of CsA concentrations, in the paired samples, taken simultaneously from both peripheral vein and indwelling catheter sites; the difference in CsA concentrations 24 h before and after the transition time between i.v. and oral treatments were determined. All evaluations were performed using the software package SAS version 8. A P-value of less than 0.05 was considered to imply statistical significance.

Results

Patients

Between April 2002 and November 2003, a total of 33 patients were included in this study. One child was excluded in the final analysis due to the child’s failure to take the
whole oral dose on the study day. The clinical characteristics are summarized in Table 1. There were 9 girls and 23 boys with a median age of 7.1 years (range: 0.8-15.6). The body weights ranged from 9 to 74.7 kg. The mean number of days until conversion from i.v. to oral CsA was 25.3 ± 6.2 days after the stem cell infusion. Twenty-one patients received soft gelatin capsules, and 11 patients were given the oral solution. Of the 32 patients who were enrolled in the study, two patients did not provide concurrent samples from the peripheral veins and indwelling catheter lines.

Pharmacokinetic parameters
The mean concentration for CsA in the whole blood versus time for 32 patients, is illustrated in Figure 1; the related pharmacokinetic data is summarized in Table 2. The interindividual variation in lag time (Tlag) was 60.4% and estimated bioavailability varied between 12.2 and 69.5% (mean 43.1 ± 14.4%). After oral administration, the time to peak concentration (Tmax) was between 0.4 and 3.6 h (mean 1.9 ± 0.8 h) after conversion. The volume of distribution ranged from 750 to 4884.9 ml/kg (mean 1871.3 ± 823 ml/kg). The mean for the total clearance was 436.9 ± 124.1 ml/h/kg and the half-life was 3.1 ± 1.2 h.

Comparison of pharmacokinetic absorption parameters by formulations
Patient characteristics for the two groups according to formulations and comparison of their pharmacokinetic absorption parameters are shown in Table 3. There were no significant differences between CsA soft gelatin capsules and solution in oral absorption for measurements evaluated such as Tlag, Bioavailability, Cmax and Tmax.

Comparison of pharmacokinetic parameters by body weight
The patient characteristics for the three groups divided by body weight (<20, 20-40 and >40 kg) are presented in Table 4. None of the comparisons between parameters studied were significant except for drug clearance. Mean clearance (CL) ± s.d. was 480.9 ± 103.7, 414.9 ± 137.1 and 320 ± 51.8 ml/h/kg in patients <20, 20-40 and >40 kg of body weight, respectively. The CsA CL of younger children was significantly greater than for older children (P = 0.044).

Comparison of concentrations in samples obtained simultaneously from different sites
Comparisons of CsA levels were determined from paired specimens taken simultaneously via the peripheral vein and

![Figure 1 Mean whole blood concentration-time profile for all patients (n = 32).](image)

Table 2  Summary of CsA pharmacokinetic parameters (n = 32)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tlag (h)</th>
<th>Ka (h⁻¹)</th>
<th>F</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (h)</th>
<th>Vd (ml/kg)</th>
<th>CL (ml/h/kg)</th>
<th>Ke (h⁻¹)</th>
<th>1/τe (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1</td>
<td>5.1</td>
<td>43.1</td>
<td>1135.3</td>
<td>1.9</td>
<td>1871.3</td>
<td>436.9</td>
<td>0.3</td>
<td>3.1</td>
</tr>
<tr>
<td>s.d.</td>
<td>0.6</td>
<td>3.3</td>
<td>14.4</td>
<td>306.6</td>
<td>0.8</td>
<td>823</td>
<td>124.1</td>
<td>0.1</td>
<td>1.2</td>
</tr>
<tr>
<td>CV (%)</td>
<td>60.4</td>
<td>65.2</td>
<td>33.4</td>
<td>80</td>
<td>44.9</td>
<td>44</td>
<td>28.4</td>
<td>35.1</td>
<td>37.7</td>
</tr>
<tr>
<td>Minimum</td>
<td>0</td>
<td>0.3</td>
<td>12.2</td>
<td>527.6</td>
<td>0.4</td>
<td>759</td>
<td>128.5</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.9</td>
<td>12.8</td>
<td>99.5</td>
<td>1883.6</td>
<td>3.6</td>
<td>4884.9</td>
<td>702.2</td>
<td>0.5</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Tlag (h) = lag time; Ka (h⁻¹) = absorption rate constant; F (%) = bioavailability; Cmax (ng/ml) = peak concentration; Tmax (h) = time to reach Cmax; Vd (ml/kg) = volume of distribution; CL (ml/h/kg) = clearance; Ke (h⁻¹) = elimination rate constant; 1/τe (h) = elimination half-life.
Table 3  Clinical characteristics of study patients according to the formulation group and comparison of their pharmacokinetic absorption parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Capsule (n = 21)</th>
<th>Solution (n = 11)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>29.7 (17.5-47.7)*</td>
<td>12.4 (9.3-15.3)*</td>
<td></td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>6/15</td>
<td>3/8</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>10 (6.1-15.6)*</td>
<td>2 (0.8-4.2)*</td>
<td></td>
</tr>
<tr>
<td>Intravenous dose (mg/kg/day)</td>
<td>3.2 ± 1</td>
<td>3.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>PO dose (mg/kg/day)</td>
<td>9.9 ± 2.7</td>
<td>10.2 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Levels on the 24 h (ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before conversion</td>
<td>348.3 ± 98.4</td>
<td>343.9 ± 155</td>
<td></td>
</tr>
<tr>
<td>After conversion</td>
<td>219.7 ± 84.7</td>
<td>206.1 ± 57.8</td>
<td></td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.2 ± 0.57</td>
<td>1.2 ± 0.7</td>
<td>0.82*</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>40.9 ± 13.9</td>
<td>47.2 ± 10.2</td>
<td>0.49*</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>1157.5 ± 327.4</td>
<td>1093 ± 377.1</td>
<td>0.64*</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.8 ± 0.8</td>
<td>2.2 ± 0.9</td>
<td>0.58*</td>
</tr>
</tbody>
</table>

All data values are presented as mean ± s.d. except *Median (range)).
*There were no significant differences between pharmacokinetic parameters of the two groups using the two-sided Wilcoxon’s rank sum test.

Table 4  Clinical characteristics of study patients according to the body weight group and comparison of their pharmacokinetic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;20 kg (n = 15)</td>
</tr>
<tr>
<td>Weight median (range)</td>
<td>13.9 (9-19.9)*</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>10/5</td>
</tr>
<tr>
<td>Age median (range)</td>
<td>2.6 (0.8-6.9)*</td>
</tr>
<tr>
<td>Formulation (L/C)</td>
<td>11/4</td>
</tr>
<tr>
<td>Intravenous dose (mg/kg/day)</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>PO dose (mg/kg/day)</td>
<td>9.9 ± 2.4</td>
</tr>
<tr>
<td>T1/2(b)</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>PL (%)</td>
<td>42.3 ± 11.3</td>
</tr>
<tr>
<td>Cmax (ng/ml)</td>
<td>1219.8 ± 295.2</td>
</tr>
<tr>
<td>Tmax (ng/ml)</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>Vd (ml/kg)</td>
<td>1806.5 ± 335.9</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>480.9 ± 103.7*</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>2.7 ± 0.6</td>
</tr>
</tbody>
</table>

All data values are presented as mean ± s.d. except *Median (range)).
*There was only significant difference between CL of the three groups using the Kruskal-Wallis test (P = 0.044).

Cyclosporin A concentrations with transition from intravenous to oral administration

The average i.v. dose of CsA taken before conversion was 3.4 ± 0.9 mg/kg per day and the oral dose after conversion was on average 9.7 ± 2.5 mg/kg per day. The Cmax mean value after conversion was 1135.3 ± 340.6 ng/ml. The mean CsA concentrations 24 h before and after the i.v.-oral transition time was 332.3 ± 95.7 ng/ml and 214.5 ± 75.8 ng/ml, respectively, (P < 0.0001). The mean CsA levels 48, 72, and 96 h after transition were 287.5 ± 150.5 ng/ml, 292.6 ± 116.4 ng/ml and 272 ± 166.1 ng/ml, respectively.

Discussion

Graft-versus-host disease remains a major limiting factor for a successful result after allogeneic HSCT; it affects mortality, morbidity and quality of life. These factors are particularly significant for pediatric patients where the growing body and multiple organ systems are especially...
vulnerable to the consequences of GVHD. CsA-based immunosuppression has been the most frequently used regimen for prophylaxis and treatment of GVHD in pediatric patients undergoing HSCT, as well as for adults. However, there remains little consensus on the administration, scheduling, dosage, and monitoring of CsA. There are some limited data on the pharmacokinetics of CsA in pediatric HSCT recipients as compared to adult transplant recipients; guidelines for CsA therapy, in the pediatric population, are derived from experience with the clinical protocols used for adults. Therefore, a comprehensive understanding of the pharmacokinetics of the CsA in the pediatric HSCT recipients is needed for the development of optimal treatment protocols and valid strategies for therapeutic monitoring.

Following its oral administration, CsA is variably and incompletely absorbed in the GI tract. The mean bioavailability of CsA in adult SCT patients is reported as 34% with a range of 20–50%. The bioavailability of the oral formulation of CsA, in pediatric transplant recipients, is as highly variable as that of the adult population. In our present study, the mean bioavailability of CsA in pediatric SCT recipients was 43.1 ± 14.4%. These observed values were a little higher than those reported for pediatric renal transplant patients, 21.8% and pediatric liver transplant patients, 37.6 ± 14.5%. The reason for this difference, between kidney transplants and our study patients, may be due to age and small bowel length. Another study has reported that small bowel length is a significant factor for CsA absorption. In the report by Hopp et al., the study patients had a mean age of 1.76 (1.08–2.53); they were younger children compared to our study patients, and therefore, they may have had a lower bioavailability.

The time to reach Cmax (Tmax) in this study was 1.9 ± 0.8 h; this is similar to the values reported for other pediatric HSCT groups, 2.4 ± 1.1 h as well as for pediatric renal transplant recipients, around 1–2 h. As previous studies have reported, the Tmax for HSCT recipients has shown that absorption is delayed in comparison to that measured in solid organ allograft recipients; it is thought that the differences are due to the presence of GI inflammation caused by mucositis or GVHD. However, we conducted part of our study at a later point in the post transplant period, when GI inflammation was improved; the pharmacokinetics of CsA in HSCT recipients may correspond more closely to those for solid organ transplant recipients during the period of time studied in our investigation.

Comparison of the pharmacokinetic absorption parameters (Tmax, Bioavailability, Cmax, T75%) for the oral solution, there were no significant differences between the two formulations in renal transplant recipients or for the heart and lung transplant recipients. Our findings for pediatric HSCT recipients support previous reports concerning the bioequivalence of soft gelatine capsules and oral solution of CsA.

The total clearance of CsA tended to be greater in the pediatric transplant population when compared to the adult transplant groups and patients 0 to 10 years old had a significantly higher CsA CL than those >11 years old. In this study, mean clearance (CL) ± s.d. was 480.9 ± 103.7, 414.9 ± 137.1 and 320 ± 51.8 ml/h/kg in patients with <20 kg, 20–40 kg and >40 kg of body weight, respectively. Cyclosporin A CL of younger children was significantly greater than older children (P = 0.044).

In adults with normal renal and hepatic function, the mean elimination half-life has been reported to average between 8.4 and 27 h and in pediatric transplant patients, a shorter half-life has been reported to be between 4.2 and 7.5 h. In our pediatric HSCT recipients, the mean estimated elimination half-life was 3.1 ± 1.2 h. Owing to the shorter half-life and the higher clearance in the pediatric population, it has been suggested that dosing every 8 h may be more appropriate in children. Several pediatric solid organ transplant centers are using a three times daily dosing schedule instead of twice daily. It has been reported that the three times daily dosing schedule of CsA in children helped to avoid deleterious peak levels with less fluctuation in blood concentration; these results have been obtained without any significant change of the average concentration of CsA. The data in our study suggest that a three times daily dose is useful for pediatric HSCT recipients.

As CsA is a lipophilic molecule, it has been postulated that the drug is adsorbed onto the surface of indwelling catheters, and that it is subsequently leached off when the lines are used for blood sampling. Previous studies have shown that the levels of samples obtained from the lumen used for CsA administration, are significantly higher than those taken from a peripheral vein. In our study, the samples were obtained from an indwelling catheter lumen that was not involved in the CsA infusion; therefore, the mean concentrations for the samples taken 24 h before the transition simultaneously from a peripheral vein and the indwelling catheter, were 332.7 ± 91.8 ng/ml and 346.7 ± 121 ng/ml, respectively (P = 0.94). The results of our present observations confirm that samples should be drawn from a catheter-lumen that was not used for i.v. CsA infusion, are valid as alternative sites to a peripheral vein for accurate therapeutic drug monitoring.

A variety of CsA administration protocols have been developed and implemented at numerous HSCT centers. These practices range from a 1 h short infusion to a continuous infusion. Previous studies have shown that CsA administration as a continuous i.v. infusion may be more effective, and it is associated with less serious side effects than bolus CsA, for the prevention of GVHD in allogeneic SCT recipients. As a consequence, in some centers CsA is administered as a continuous infusion in the early period after stem cell transplantation. Thereafter, when patients were able to eat normally, usually around 25 days after HSCT, they were converted from i.v. CsA to an oral formulation. The oral formulation was administered immediately after discontinuation of the continuous infusion with the attempt to maintain CsA levels. In other studies, the mean or median Cmax have been reported as 1200–1500 ng/ml. In our study, the mean peak concentration of CsA (Cmax) after oral administration was 1135.3 ± 340.6 ng/ml, not excessive Cmax levels. Therefore, according to this observation, our administration method for the transition period is effective and practical for routine clinical use.
Additional study will further define more specific dosing intervals and conversion doses for oral treatment after continuous intravenous dosing is discontinued. Improved knowledge of doses and time intervals will allow for appropriate adjustments to be made on the basis of pharmacokinetic parameters.

References


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REVIEW

Optimizing the use of cyclosporin in allogeneic stem cell transplantation

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Cyclosporin remains the most widely used immunosuppressive agent in patients undergoing allogeneic stem cell transplantation (SCT). The increased awareness of the impact of the intensity of post-transplant immunosuppression on determining outcome after allogeneic SCT has resulted in a re-examination of whether cyclosporin is currently being optimally used in this population of patients. Recent studies in solid organ transplantation have questioned whether the use of trough levels provides the most accurate reflection of the immunosuppressive actions of cyclosporin and alternative strategies to monitor cyclosporin dosage after liver and kidney transplantation are increasingly being used. As a result there is now interest in examining whether there is scope for translating these advances into the arena of haematopoietic transplantation. In this paper, we will review the rationale underlying the current schedules for dosing and monitoring cyclosporin after allogeneic SCT and identify specific areas in which the use of cyclosporin requires re-evaluation. These include evaluation of whether patient outcome would be improved by using peak cyclosporin levels to determine dosing schedules, analysis of optimal cyclosporin dosing schedules in patients undergoing reduced intensity allografts and investigation of surrogate markers of cyclosporin’s immunosuppressive activity.

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Keywords: cyclosporin; allogeneic SCT; monitoring

Introduction

Pharmacological suppression of the donor-derived alloreactive immune response has played a central role in reducing the morbidity and mortality of graft-versus-host disease (GVHD), which still remains the major cause of toxicity after allogeneic stem cell transplantation (SCT). At the same time, it has become clear that the intensity of post-transplant immunosuppression is one of the most important determinants of relapse risk through its impact on the potency of an immunologically mediated graft-versus-leukaemia (GVL) effect. This is particularly relevant in patients undergoing allogeneic SCT using a reduced-intensity conditioning (RIC) regimen, where a GVL effect represents the dominant anti-leukaemic mechanism. Accordingly, there is now a developing interest in tailoring the intensity and duration of post-transplant immunosuppression according to the perceived risk of GVHD and disease relapse in an individual patient.

In the past few years considerable refinement has occurred in the pharmacologically mediated immunosuppressive strategies utilized in recipients of solid organ transplants. This has occurred through the use of more precise methods of monitoring established agents, principally cyclosporin (CsA), which have been shown to reduce the risk of graft rejection in the setting of both kidney and liver transplantation. This experience suggests that there may be scope for translating the advances that have been made in the field of solid organ transplantation to recipients of allogeneic haematopoietic stem cells. This review will focus on the basis for current immunosuppressive strategies in patients undergoing allogeneic haematopoietic SCT and discuss whether there is room for improving both the monitoring and the delivery of pharmacologically mediated immunosuppression in this population of patients.

The role of cyclosporin and other immunosuppressants in graft-versus-host disease prophylaxis

Cyclosporin has become the most widely used agent for the prevention of GVHD in patients undergoing allogeneic SCT after initial studies by Powles9 demonstrated that its administration, in conjunction with methotrexate, resulted in a substantial reduction in the incidence and severity of acute GVHD and had a positive impact on survival. This pioneering work was subsequently confirmed by randomized controlled trials from the Seattle group.2 In recent years a number of newer immunosuppressants have entered clinical practice and have been compared to the standard CsA plus methotrexate regimen (Table 1).5 The maximum experience has been gained with tacrolimus, a calcineurin inhibitor that is chemically unrelated to CsA. Two large randomized phase III trials of tacrolimus plus methotrexate versus CsA plus methotrexate have been published, both of which showed a significantly reduced incidence of
Table 1  Phase III clinical trials comparing newer immunosuppressants with cyclosporin-based schedules for GVHD prophylaxis

<table>
<thead>
<tr>
<th>Reference</th>
<th>Comparison</th>
<th>Population</th>
<th>Number of patients</th>
<th>cGVHD (grade II-IV)</th>
<th>cGVHD</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratanatharatorn et al.</td>
<td>Tac + MTX vs CSA + MTX</td>
<td>Sibling allografts</td>
<td>329</td>
<td>32% (Tac/MTX) vs 44% (CSA/MTX) (P = 0.01)</td>
<td>56 vs 49% (NS)</td>
<td>47 vs 57% (P = 0.02) at 2 years</td>
</tr>
<tr>
<td>Nadal et al.</td>
<td>Tac + MTX vs CSA + MTX</td>
<td>MUD allografts</td>
<td>180</td>
<td>56% (Tac/MTX) vs 74% (CSA/MTX/TX) (P = 0.0002)</td>
<td>76 vs 70% (NS)</td>
<td>54 vs 50% (NS) at 2 years</td>
</tr>
<tr>
<td>Hirokata et al.</td>
<td>Tac vs CSA</td>
<td>Sibling and MUD allografts</td>
<td>136</td>
<td>17.5% (Tac) vs 48% (CSA) (P &lt; 0.001)</td>
<td>47.3 vs 47.8% (NS)</td>
<td>63 vs 65% (NS) but more relapses in tacrolimus sibling allograft group</td>
</tr>
<tr>
<td>Bolwell et al.</td>
<td>CSA/MMF vs CSA + MTX</td>
<td>Sibling allografts</td>
<td>40</td>
<td>48% (CSA/MMF) vs 37% (CSA/MTX) (NS)</td>
<td>63 vs 64% (NS)</td>
<td>52 vs 68% (NS) at 9/12 but MMF arm less toxic</td>
</tr>
</tbody>
</table>

Abbreviations: CSA = cyclosporin; Tac = tacrolimus; aGVHD = acute GVHD; cGVHD = chronic GVHD; MTX = methotrexate; MMF = mycophenolate mofetil; MUD = matched unrelated donor.

Acute GVHD in patients receiving tacrolimus. However, there was no difference in the incidence of chronic GVHD in either study and in the study involving patients undergoing a sibling allograft, overall survival was in fact worse in the tacrolimus plus methotrexate arm. As yet, only one small randomized phase III trial of mycophenolate mofetil as GVHD prophylaxis has been published. Mycophenolate mofetil in combination with CSA resulted in similar rates of GVHD and a comparable overall survival to cyclosporin plus methotrexate but was better tolerated in terms of faster engraftment and less mucositis. Encouraging results have also been reported with sirolimus, a macrocyclic lactone immunosuppressant that is structurally similar to tacrolimus.

Despite these developments, the combination of CSA and methotrexate remains the standard approach by GVHD prophylaxis in the majority of transplant centres. However, there is still uncertainty as to the optimal schedule for dosage, administration and monitoring of CSA. A survey of 87 centres by the European Group for Blood and Marrow Transplantation showed inconsistency in both timing of CSA infusion and variations in the range of dose from 1 to 20 mg/kg/day. Reference ranges also varied widely between 100 and 1000 μg/l. There was also a lack of standardization in assay techniques with both whole blood and serum being used for measurement and a number of different assay methods being employed. A recent survey of UK practice involving 19 transplant centres produced results similar to those described above revealing target concentrations for CSA ranging from 85 up to 800 μg/l.

Although there is clearly uncertainty surrounding the optimal use of CSA in the setting of GVHD prophylaxis, accumulating data attest to the potential clinical benefits of close attention to CSA dosing schedules and monitoring. In an important study in which patients undergoing a sibling allograft were randomized to 0 or 5 mg/kg/day CSA, Bacigalupo demonstrated a significantly higher rate of acute and chronic GVHD in patients receiving 1 mg/kg CSA. This was offset by a decreased risk of relapse in patients receiving low-dose CSA, resulting in improved disease-free survival in this group of patients. Other groups have shown that close monitoring of CSA can produce benefits in terms of a reduced incidence of acute GVHD while extended CSA administration has the capacity to reduce the risk of chronic GVHD. More recently it has been demonstrated that post-transplant immunosuppression using CSA permits durable engraftment of allogeneic stem cells using a non-myeloablative regimen. In the setting of allografts performed using a RIC regimen, where the dominant anti-tumour effect of the allograft is exerted by an immunologically mediated GVL effect, CSA is rapidly tapered post transplant. However, the optimal dosing schedule and duration of CSA administration in patients undergoing RIC allografts and the impact on relapse and GVHD are unknown, and requires urgent further study. This is particularly true in patients in whom T-cell depletion using alemtuzumab or anti-thymocyte globulin is used, where relapse is the most important cause of treatment failure and reduction in CSA dose intensity may represent an important, and largely unexplored, approach towards improving outcome. Clearly therefore CSA plays a critical role in determining the outcome of allogeneic SCT using both myeloablative and RIC regimens and this has led to renewed interest in refining the current techniques available for monitoring both the blood level and the pharmacological activity of CSA after allogeneic SCT.

Pharmacology of CSA

Cyclosporin was first introduced into clinical practice in 1978 and has become the cornerstone of immunosuppression in the settings of solid organ (renal, hepatic and cardiac) and SCT. Cyclosporin exerts its immunosuppressive effects through suppression of calcineurin-mediated T-lymphocyte activation and accumulating evidence demonstrates that peak levels of CSA correlate best with the degree of calcineurin inhibition achieved. Consequently, factors determining peak as well as trough levels of CSA may significantly impact on the degree of immunosuppression produced by patients receiving this agent post transplant. This is of particular importance given that CSA pharmacokinetics are highly variable, with factors such as transplant type, patient age and concurrent drug therapy all influencing blood concentrations of the drug. Variable absorption of oral CSA is well documented, and can result in marked differences in bioavailability between patients. In the SCT setting, absolute bioavailability of oral CSA may vary from 20 to 50%. However, in the first few days post transplant, mucositis caused by the pre-transplant conditioning regimen impairs absorption of CSA from the upper gastrointestinal tract. In addition,
oral mucositis can make it difficult for the patient to tolerate an oral formulation. Consequently, administration of the drug by the intravenous route until mucositis has resolved is standard practice in almost all transplant centres. Cyclosporin is extensively metabolized, with the liver being the main site of metabolism. Age and hepatic function can impact on CsA clearance and drugs commonly used in the setting of SCT such as phenytoin, itraconazole, fluconazole and macrolide antibiotics can also influence the metabolism of CsA. For example, phenytoin can reduce CsA concentrations by 50% via induction of hepatic microsomal enzymes.

Given the marked interpatient variability in CsA pharmacokinetics and the importance of maintaining a balance between underdosing (and thus increasing the risk of GVHD or rejection) and overdosing (and thus increasing the risk of toxicity), blood-level monitoring has for many years been the standard practice for patients receiving CsA in the solid organ and SCT settings. Trough-level monitoring was first introduced into clinical practice in the early 1980s and despite its widespread usage over the subsequent two decades, there has remained a degree of uncertainty as to whether this approach is the most appropriate way of performing drug monitoring of CsA. In the setting of GVHD prophylaxis, a number of authors have demonstrated an inverse relation between trough CsA levels and incidence of GVHD. However, other authors have failed to demonstrate a significant relationship between trough concentrations and aGVHD and conflicting findings have also been reported when studying the association between CsA levels and the risk of nephrotoxicity. Given that there appears to be a lack of a strong association between trough CsA concentrations and measures of both efficacy and toxicity in the SCT setting, it is instructive to focus on the solid organ transplantation literature, where the issue of how best to monitor CsA has generated significant interest in the past two decades.

Cyclosporin monitoring in the setting of solid organ transplantation

Early studies using oral CsA in solid organ transplantation suggested that trough concentrations correlated with clinical outcomes, both in terms of rejection and nephrotoxicity and also that the correlation with trough levels was better than with the maximum concentration (Cmax). Consequently, trough-level monitoring became the standard practice, although concern remained that this technique was still an imperfect tool. This was illustrated by data showing that the rates of acute rejection and nephrotoxicity in patients with trough CsA concentrations within a defined therapeutic range remained 59 and 63%, respectively. Subsequent work showed that trough CsA levels did not correlate with drug exposure as estimated by the area under the concentration time curve (AUC), and others demonstrated that the correlation between trough levels and clinical outcomes was relatively poor in the settings of renal, liver and heart transplantation.

These concerns led to increased interest in developing alternative monitoring strategies for CsA and a number of studies demonstrated the benefit of AUC monitoring as a superior indicator of drug exposure and clinical events in the setting of renal transplantation. However, full AUC monitoring is a time consuming and relatively impractical process, so efforts were made to simplify its determination. This led to the development of sparse-sampling algorithms, which used two or three time points as surrogate markers for exposure to CsA and were found to correlate with AUC. The logical progression of this approach was to find a single time-point measure which correlated best with AUC and a number of studies showed that a sample taken 2h after an oral dose of CsA (C2) was the most accurate single marker of exposure with a correlation coefficient of >0.80 across a variety of transplant populations.

In addition to demonstrating an association between C2 and AUC, it was also noted that there was good correlation between C2 and Cmax. The emerging evidence that calcineurin inhibition correlated well with CsA levels, with maximal inhibition occurring during the period of highest CsA concentration, provided a further pharmacodynamic argument for utilizing C2 monitoring in the post-transplant setting.

A number of trials have employed C2 monitoring in solid organ transplantation and demonstrated superior outcomes in terms of rejection rates and acute toxicities compared to trough-level monitoring. This has led some to conclude that C2 monitoring is now the optimal method of monitoring oral CsA in the setting of de novo renal and hepatic transplants. Consequently, C2 monitoring is being increasingly employed in the management of solid organ recipients with a recent survey of renal transplant centres finding that almost half the respondents were now measuring C2 concentrations.

Improving strategies for immunosuppression after allogeneic haematopoietic stem cell transplantation

In the past 5 years, the monitoring of oral CsA in the setting of solid organ transplantation has undergone significant change, based on concerns regarding the validity of the traditional system of trough-level monitoring. Given the marked interpatient variability in the absorption of oral CsA and the findings that the peak CsA concentration correlates well with maximum inhibition of calcineurin, the move towards C2 monitoring seems logical from both pharmacokinetic and pharmacodynamic perspectives. In the SCT setting, where it is clear that our current practice of trough-level monitoring still results in unacceptably high levels of aGVHD, there is scope to explore the potential of monitoring the drug (both i.v. and oral) via alternative measures of exposure or activity. For example, it will be important to determine whether the AUC or peak level, rather than the trough level, provides a more accurate measure of post-transplant immunosuppression. Importantly, whatever pharmacokinetic marker is employed, there remains the concern that this may not accurately reflect the functional activity of the drug, and this has led a number of groups to investigate the use of calcineurin activity as a marker of immunosuppression after allogeneic SCT. Significantly, it has been reported that the risk of
developing of acute GVHD correlates with the degree of calcineurin inhibition in the mononuclear cells of patients in the first 2 months post transplant and this potentially important observation requires validation in a larger cohort of patients. There is also emerging evidence that there is significant inter-patient variation in the degree of calcineurin inhibition produced by a consistent dose of CsA, emphasizing the importance of correlating biological measures of calcineurin inhibition with clinical outcome.

Conclusions and future directions

In conclusion, it is clear that CsA plays a central, and sometimes underestimated, role in optimizing outcome after allogeneic SCT. This is particularly the case in the setting of RIC allotrafts, where CsA's impact on the potency of the alloreceptove response plays a critical role in modulating the GVL effect post allotraft. However, despite its importance in determining outcome, there is little consensus concerning how CsA is administered or monitored. In light of the emerging evidence from solid organ transplants that peak levels of CsA may determine clinical alloreactivity, a reassessment of monitoring strategies with respect to GVHD and disease relapse is now important in the setting of both myeloablative and RIC allotrafts. For example, we would propose that studies are undertaken to address the issue of whether peak-level monitoring may be superior to trough-level monitoring in SCT patients receiving both i.v. and oral cyclosporin. In addition, in light of the fact that relapse remains the major cause of treatment failure after RIC allotrafts, randomized controlled studies examining both the dose intensity and duration of CsA administration after allogeneic SCT are now indicated. These studies are of particular importance given the current limitations in intensifying the preparative regimen without compromising the ability of RIC allotraft protocols to extend the age at which allogeneic SCT can be safely performed. It is therefore clearly time to re-evaluate the optimal use of one of the oldest drugs in the transplant armamentarium.

- Cyclosporin dose intensity is an important and readily manipulable determinant of the incidence of GVHD post allogeneic SCT and plays a particularly important role in determining outcome after RIC allotrafts.
- Dose intensity of cyclosporin should be adjusted according to the perceived risk of disease relapse and GVHD.
- Trough levels of cyclosporin correlate poorly with the degree of immunosuppression and outcome in solid organ transplantation.
- In the setting of solid organ transplantation there is increased interest in the use of peak-level monitoring of oral cyclosporin.
- Optimal strategies for monitoring cyclosporin dose intensity in the setting of stem cell transplantation remain to be defined and require randomized studies in the setting of both myeloablative and RIC allotrafts.

References


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Cyclosporine short infusion and C2 monitoring in haematopoietic stem cell transplant recipients

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Blood concentrations of cyclosporine A (CsA) ≥ 800 µg/l measured 2h post-dosing, the C2 concentration, is necessary to obtain a maximal pharmacological effect and correlates well with transplant-related complications such as transplant rejection and toxicity. In an open crossover study CsA blood levels were measured during 24h to generate a pharmacokinetic profile on days 1, 8 and 15 after starting CsA infusion in 21 haematopoietic allogeneic stem cell transplant recipients who were receiving intravenously CsA 3mg/kg/day either by continuous infusion or by 2h infusion given every 12h. C2 levels after the 2h infusion correlated better than C1 or C3 levels with the area under the concentration-time curve from 0 to 4h (r² = 0.62). C3 levels ≥ 800 µg/l were also achieved for 20 out of 24 (83%) of cases after the 2h infusion of CsA without any increase of CsA-related toxicity but for only three of the 23 patients (13%) after continuous infusion. Therefore, we recommend CsA infusions in 2h during transplant and perform C2 monitoring to obtain therapeutic C2 levels ≥ 800 µg/l.

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Keywords: cyclosporine; HSCT; C2; AUC; safety

Introduction

Cyclosporine A (CsA)-alone or in combination with methotrexate is widely used for graft-versus-host disease (GVHD) prophylaxis among haematopoietic allogeneic stem cell transplant (HSCT) recipients.1 CsA binds to the cyclophilin of lymphocytes resulting in calcineurin inhibition which, in turn, leads to reduced interleukin-2 (IL-2) levels and inhibition of T-lymphocyte proliferation. The CsA acts in a concentration-dependent rather than a time-dependent fashion and it has been shown that the greatest pharmacodynamic effect occurs within the first 2h of exposure (C2) at peak CsA levels of 800–2285 µg/l which result in 70–96% calcineurin inhibition and maximum suppression of IL-2 release from T cells.2,3 C2 monitoring has been adopted for renal, liver and heart transplant recipients as the 2h peak concentration correlates well with the area under the concentration-time curve (AUC) from 0 to 4h after giving the drug and that this predicts the occurrence of acute rejection and nephrotoxicity. C2 monitoring is also associated with clinical benefits like a reduction in graft rejections after solid organ transplantation.4,5 Surprisingly, data on pharmacokinetics and target C2 levels are lacking in HSCT patients. Moreover, there is no consensus about how CsA should be administered with some centres giving 1 or 2 intermittent infusions and others continuous 24h infusion.6 Hence, we decided to determine whether administering CsA at a daily dose of 3 mg/kg/day intravenously (i.v.) in 2h (short infusion) twice-daily achieved C2 blood levels of at least 800 µg/l and whether it was feasible and safe.

Patients and methods

Patients

From June 2001 till August 2002, 22 patients undergoing allogeneic HSCT for various haematological malignancies after myeloablative preparative regimens gave their informed consent to participate in the study. Subjects were eligible if they were aged between 18 and 65 years, were to receive an HLA-matched, MLC-negative, partial T-cell depleted (98%) allogeneic HSCT, had never been exposed to cyclosporine before. Patients were excluded if they had a body mass index (BMI) above 30 kg/m², had received systemically active azoles within the 2 weeks before HSCT or had elevated serum creatinine levels in excess of 220 µmol/l at study entry. On admission a polyurethane quadruple lumen catheter (Arrow-Howes Quad-Lumen Central Venous Catheterization Set with Blue FlexTip Catheter, Arrow International, Reading, USA) was inserted in the right or left subclavian vein. Antimicrobial prophylaxis and therapy was given in accordance with our standard of care. All patients received ciprofl oxacin 500 mg b.i.d. orally as prophylaxis. This was stopped and changed to meropenem 1 g t.i.d. i.v. the day after transplant and continued until the end of neutropenia. Fluconazole 200 mg
once daily was only given to patients shown to be colonized with susceptible *Candida* species. No other azole antifungal agents were given as prophylaxis.

**Study design**

The study was approved by the Ethics Committee and was an open crossover study in which half of the trial subjects were to receive cyclosporine 1.5 mg/kg/day by continuous infusion over 12 h twice daily for the first 7 days then by a 2-h infusion of the same dose twice daily for the next 7 days (Group A). The other half of the trial subjects were to receive the same dose of cyclosporine by a 2-h infusion twice daily for the first 7 days then by continuous infusion over 12 h twice daily for the next 7 days (Group B).

A random allocation list was prepared by the pharmacist before inclusion of the first patient started and each individual treatment allocation was placed in a series of sequential, numbered sealed envelopes. The pharmacist was contacted once a patient fulfilled all the inclusion and exclusion criteria and the next consecutive envelope was opened to reveal the treatment group that had been allocated. Administration of CsA began on the day of transplant (study day 1) with the drug being given exclusively via the median lumen twice daily at 0900 and 2100 hours.

**Cyclosporine blood level monitoring: C₀ and AUC**

After discarding the first 5 ml of blood, blood samples of 10 ml were drawn via the distal lumen of the catheter on study days 1, 8 and 15 just before the first infusion of CsA ( trough level ) and 1, 2, 3, 4, 6, 8, 12 and 24 h after to obtain a pharmacokinetic profile on each of these days, according to the method described by Luke et al. Prom. CsA blood concentrations were measured using a monocular whole blood assay (TDX Abbott Laboratories, North Chicago, IL, USA). This assay detects 25-1500 μg/l of CsA with a coefficient of variation of less than 4%.

**Safety**

Laboratory measurements were performed daily and included creatinine, electrolytes, glucose and liver function tests. Serum protein and magnesium were measured twice weekly. Serum creatinine was also scored using the method described by Lindholm et al. which considers an increase of the serum creatinine value greater than 20% within a 7-day period as nephrotoxicity. Concomitant medication and parenteral fluid administration were recorded. Vital signs and body weight were noted at recruitment and four times daily until day 28. Furosemide was given when the body weight increased by >2 kg over the admission weight. The Common Terminology Criteria for Adverse Events v3.0 (CTO) were used to score adverse events. Each patient was followed for up to 3 months after starting CsA to enable registration of major transplant-related complications as acute GVHD according to the Glucksberg criteria or death.

**Statistical analysis**

The Student’s two-tailed t-test was used to compare numerical data and the χ²-test was used to compare frequencies of toxicity as scored by the CTC criteria between the two groups. This test was also used to compare differences in the occurrence of direct CsA infusion-related side effect that is all blood samples taken at the correct time and no CsA levels exceeding 1500 μg/l. The AUC₀₋₉h and its relationship to CsA levels at 1 h ( C₁ ), 2 h ( C₂ ), and 3 h ( C₃ ) after starting the 2-h infusion was analysed using Prism 4 software (Graphpad Software Inc). P-values <0.05 were considered statistically significant.

**Results**

One patient was excluded because of prior azole drug usage. The characteristics at study entry of each group were similar and age, protein levels and BMI were comparable (Table 1).

**Cyclosporine blood level monitoring: C₀ and AUC₀₋₉h**

In group A, nine out of 33 profiles were excluded from analysis because CsA was not infused exactly as described in the study protocol (n=9) or because of missing blood samples (n=4). In group B, seven out of 30 profiles were excluded because CsA was not infused exactly as described.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group A (n=11)</th>
<th>Group B (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (s.d.)</td>
<td>43 (14)</td>
<td>45 (13)</td>
</tr>
<tr>
<td>Male patients (%)</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Diagnoses (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute lymphoid leukaemia</td>
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<tr>
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<tr>
<td>Multiple myeloma</td>
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</tr>
<tr>
<td>Myelo dysplastic syndrome</td>
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<td>0</td>
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<tr>
<td>Conditioning regimen (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy + TBI</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Cy + ATG + (TBI or Bu)</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Cy + Bu + (TBI or Bu)</td>
<td>4</td>
<td>2</td>
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<tr>
<td>Stem cell donor (%)</td>
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<tr>
<td>HLA identical sibling</td>
<td>8</td>
<td>8</td>
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<tr>
<td>HLA-matched VUD*</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Body mass index, mean (s.d.)</td>
<td>26.2 (2.3)</td>
<td>24.9 (2.5)</td>
</tr>
<tr>
<td>Total protein (g/dl), mean (s.d.)</td>
<td>68 (6.3)</td>
<td>65 (5.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Complied with the protocol* (%)</th>
<th>Day 1</th>
<th>Day 8</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complied</td>
<td>8</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Exceeded</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Abbreviations: Cy = cyclophosphamide; TBI = total body irradiation; ATG = antithymocyte globulin; IDA = idarubicin; Bu = bursaphen; VUD = voluntary unrelated donor.

*Cytoxan given according to the protocol and samples available for at least one analysis.

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Bone Marrow Transplantation
in the study protocol (n = 5) or because of missing blood samples (n = 2). To illustrate the difference of profiles generated during CsA short infusion and continuous infusion respectively, two evaluable profiles are shown in Figure 1.

In total, eight pharmacokinetic profiles were obtained after continuous infusion from patients in group A on day 1 and a further eight and seven, respectively, were obtained from patients in group B on days 8 and 15. Levels exceeding 800 μg/L were found in only three of 23 cases. By contrast 20 out of 24 available pharmacokinetic profiles following 2 h infusion showed a CsA ≥ 800 μg/L (eight on day 1 from group B and nine and seven, respectively, on days 8 and 15 from Group A). The four patients who received CsA short infusions and did not achieve CsA levels > 800 μg/L had CsA levels of 559, 684, 773 and 784 μg/L.

Fifteen profiles were available for CsA-analysis for six, six and three patients, respectively, on days 1, 8 and 15 after the 2-h infusion. CsA levels of CsA correlated better than did either C1 or C3 with the AUC CsA 24 h.

**Concomitant drugs**
Six patients in group A and four in group B were given fluconazole as prophylaxis. One patient in group A and two in Group B were given metoclopramide. No other drugs known to interact with cyclosporine were given during the study period.

**Incidence of acute GVHD**
Fifteen out of 21 (71%) patients developed acute GVHD of the skin, from which eight had grade 2 or higher.

**Safety**
Infusion-related side effects. Patients who received CsA in 2-h infusions did not experience significantly more adverse events than those who were given the drug continuously (Table 2). In the second study week 5 patients who had received short infusions and 1 patient given continuous infusion reported a burning sensation of hands or feet.
Table 2 Toxicity during day 1 until 28

<table>
<thead>
<tr>
<th></th>
<th>Days 1-7</th>
<th></th>
<th>Days 8-14</th>
<th></th>
<th>P-value</th>
<th>Days 1-7</th>
<th></th>
<th>Days 8-14</th>
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<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>Continuous</td>
<td>2-h</td>
<td>P-value</td>
<td>Continuous</td>
<td>2-h</td>
<td>P-value</td>
<td>Continuous</td>
<td>2-h</td>
<td>P-value</td>
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<tr>
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<td>Hypertension CTC*</td>
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<td>0.33</td>
<td>1</td>
<td>2</td>
<td>0.59</td>
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<tr>
<td>Days of furosemide (n.s.d.)</td>
<td>0.36 (0.50)</td>
<td>0.10 (0.32)</td>
<td>0.17</td>
<td>0</td>
<td>1.09 (1.70)</td>
<td>0.059</td>
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<tr>
<td><strong>Electrolyte disturbances</strong></td>
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<tr>
<td>Days potassium ≥4.7 mmol/l (n.s.d.)</td>
<td>1.36 (1.39)</td>
<td>1.70 (2.16)</td>
<td>0.69</td>
<td>2.80 (3.05)</td>
<td>1.09 (1.58)</td>
<td>0.14</td>
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<tr>
<td>Magnesium mmol/l (n.s.d.)</td>
<td>0.67 (0.675)</td>
<td>0.70 (0.655)</td>
<td>0.44</td>
<td>0.65 (0.985)</td>
<td>0.65 (0.055)</td>
<td>0.94</td>
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<td><strong>Nephrotoxicity</strong></td>
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<tr>
<td>Creatinine rise &gt;20% within 7 days. No. of days (n.s.d.)</td>
<td>0.091 (0.30)</td>
<td>0.20 (0.42)</td>
<td>0.51</td>
<td>1.9 (1.52)</td>
<td>2.0 (1.79)</td>
<td>0.89</td>
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<td><strong>Hepatotoxicity</strong></td>
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<tr>
<td>Icterus and elevated total bilirubin*</td>
<td>2</td>
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<td>0.16</td>
<td>0</td>
<td>2</td>
<td>0.16</td>
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<tr>
<td>Total bilirubin CTC</td>
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<td>10</td>
<td>10</td>
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<td>1</td>
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<td>ALAT CTC</td>
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<td>0.53</td>
<td>3</td>
<td>3</td>
<td>0.89</td>
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<tr>
<td>Alkaline phosphatase CTC</td>
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<td>1</td>
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<td>0.94</td>
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<tr>
<td>γGT CTC</td>
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<td>1</td>
<td>0.031*</td>
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<td>4</td>
<td>0.86</td>
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<td>Headache</td>
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<td>1</td>
<td>0.94</td>
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<tr>
<td>Burning sensation hand and/or foot</td>
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<td>2</td>
<td>0.48</td>
<td>1</td>
<td>5</td>
<td>0.072</td>
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<tr>
<td>Tendon</td>
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<td>Mental disturbance</td>
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<td>4</td>
<td>0.034*</td>
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</table>

*If used, CTC grade 2 or higher was defined as toxicity.

**During the icteric phase, all patients developed total bilirubin CTC grade 3 or 4.

*P = <0.05.

during CsA administration. This was not a significant difference (P = 0.072) although it was the reason why one patient declined further short infusions of the drug.

Noninfection-related side effects. There were no significant differences between the 2 h administration and continuous infusion in nephrotoxicity or hepatotoxicity except that γGT, which was significantly higher (CTC-NCIC toxicity grade 2) during the first week of study (P = 0.031) in the group that received CsA continuously.

One patient in Group A experienced severe neurotoxicity CTC grade 2 that progressed to toxic-metabolic encephalopathy starting from day 8. This patient died on day 37 of respiratory insufficiency and acute GVHD. Another patient in Group B was readmitted on day 28 because of acute kidney failure (creatinine 575 μmol/l), which disappeared after interrupting treatment with CsA. In the second week of the study catheter-related venous subclavian thrombosis CTC grade 2 was diagnosed in four cases all in Group A (P = 0.034). This was unlikely the result of the short infusion of CsA though no other plausible explanation could be found.

Discussion

This study shows that CsA short infusions can safely induce C2 concentrations ≥800 μg/l in HSCT recipients. Moreover, C2 correlates well with the AUC0-48, which is a good indicator of exposure. Although CsA has been used routinely for prevention of GVHD (and graft rejection) in solid organ and stem cell transplantations since the early 1980s, it is still not known which dose and duration of treatment is optimal for HSCT recipients. Runtu et al. reported wide variation regarding CsA usage for prophylaxis and treatment of GVHD in a survey of 87 European Bone Marrow Transplant Centres. As a case in point, they found an initial median dose of 3 mg/kg CsA given i.v. with a range of 1–20 mg/kg. As previously stated, maximal suppression of IL-2 release from T-cells occurs within the first 2 h after CsA administration. Therefore, achieving a CsA peak concentration by infusing CsA 3 mg/kg in 2 h twice daily seems more rational than giving the drug by continuous infusion of 24 h.

The narrow therapeutic index of CsA makes it important to evaluate the real CsA dose a patient is exposed to. This is best represented by the AUC0-48, which in turn is most accurately predicted by CsA C2 levels. In renal, liver and heart transplant patients, there is already increasing evidence that C2 not only correlates well with the AUC0-48, and that C2 monitoring to ensure adequate levels resulted in lower incidences of rejection and nephrotoxicity. Unfortunately, there are no data to our knowledge about C2 monitoring among HSCT recipients. In our study, short infusion of CsA resulted in C2 levels ≥800 μg/l in 20 of 24 (83%) patients.
CsA samples were drawn through a multiple lumen polyurethane CVC which we had shown previously to be as reliable as drawing samples by venipuncture. 17

The incidence of acute GVHD of the skin we noted in this study is to be expected in T-cell-depleted allogeneic HLA-matched HSCT. However, analogous to the reduction of graft rejection and toxicity in kidney transplant patients, a reduction in GVHD in HSCT recipients can be expected if CsA dose will be adjusted to C2 levels.

This pilot study showed that C2 levels \( \geq 800 \mu g/l \) can be achieved by giving 3 mg/kg/day CsA in two divided doses i.v. in a 2 h infusion and that there was no significant difference in the incidence side effects or toxicity between the this mode of administration and the same dose given over 24 h by continuous infusion. We have now adopted this scheme and recommend increasing the dose of CsA if C2 levels of \( \geq 800 \mu g/l \) are not achieved as this correlates best with good exposure. However further investigation is clearly necessary in order to establish if C2 monitoring among HSCT recipients leads to the improved engrafting, decreased rejection, lower incidence of GVHD and less toxicity as has been shown for kidney, liver and heart transplant recipients.

Acknowledgements

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References


Graft-versus-host disease

A prospective randomized trial comparing cyclosporine and short course methotrexate with cyclosporine and mycophenolate mofetil for GVHD prophylaxis in myeloablative allogeneic bone marrow transplantation

B Bolwell, R Sobecks, B Pohlman, S Andresen, I Rybicki, E Kuczkowski and M Kalaycio

Department of Hematology and Medical Oncology, Department of Biostatistics and Epidemiology, Taussig Cancer Center, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Summary:
Cyclosporine (CSP) and short course methotrexate (MTX) have been the gold standard for GVHD prophylaxis for decades. Problems associated with MTX include increased time to hematopoietic engraftment, mucositis, and other organ toxicities. The combination of CSP with mycophenolate mofetil (MMF) has been used successfully for the prevention of graft rejection and GVHD in nonmyeloablative transplantation. We performed a prospective randomized trial comparing CSP and MTX with CSP and MMF in myeloablative (busulfan based) allogeneic 6/6 matched sibling bone marrow transplantation (BMT). The group receiving MMF (n=21) had significantly less severe mucositis than did the group receiving MTX (n=19) (21 vs 65%, P = 0.008). Median time to neutrophil engraftment was more rapid in the MMF group (11 vs 18 days, P<0.001). The incidence of acute GVHD, as well as 100 day survival, was similar for both groups. The reduced toxicity of the CSP and MMF arm resulted in premature study closure. We conclude that a GVHD prophylaxis regimen of CSP and MMF after a myeloablative allogeneic preparative regimen is associated with faster hematopoietic engraftment, decreased incidence of mucositis, similar incidence of aGVHD, and comparable survival as compared to CSP and MTX. Bone Marrow Transplantation (2004) 34, 621–625. doi:10.1038/sj.bmt.1704647
Published online 9 August 2004
Keywords: GVHD; allogeneic bone marrow transplantation; mycophenolate; methotrexate

MATERIALS AND METHODS

Study design

Eligible patients included those with hematologic malignancy who were appropriate candidates for a myeloablative allogeneic BMT. All patients were required to have a 6/6 HLA matched related donor. All donors were required to undergo a bone marrow harvest. This protocol was reviewed and approved by the Cleveland Clinic Foundation’s Institutional Review Board, with informed consent obtained from all patients. Patients were prospectively randomized 1:1 to receive either CSP plus MTX or CSP plus MMF for GVHD prophylaxis. All patients received 300 mg/m² CSP by continuous infusion intravenously daily from day -1 until hematopoietic engraftment, and thereafter orally attempting to maintain a therapeutic trough level of 200. Patients randomized to MTX received a dose of 5 mg/m² intravenously on day +1, +3, +6, and +11 after allogeneic BMT. Patients randomized to MMF received 500 mg three times daily either intravenously or orally depending on the patient's ability to tolerate oral
medication with the first dose administered on day +1 after BMT and continuing until day +100.

Study end points included the incidence of acute GVHD, severity of mucositis, time to engraftment of neutrophils and platelets, and 100-day survival.

Definitions

Patients were divided into 'good risk' and 'poor risk' based on disease status at the time of transplant. Good risk was defined as any leukemia in complete remission, chronic myelogenous leukemia in chronic phase, chemotherapy-sensitive follicular lymphoma, aplastic anemia, or chemotherapy sensitive chronic lymphocytic leukemia. Poor risk was defined as any other disease status, including chemotherapy insensitive malignancies and refractory disease.

Bone marrow transplant preparative regimen and supportive care

The preparative regimen was busulfan and cyclophosphamide (busulfan 16 mg/kg orally, cyclophosphamide 120 mg/kg). All patients received allogeneic bone marrow from donors undergoing a bone marrow harvest. Patients were hospitalized for the delivery of high-dose chemotherapy and discharged after adequate hematological recovery. Platelet or RBC transfusions were routinely administered for a platelet count <15 x 10^9/l or hemoglobin <8.5 g/dl, respectively. All blood products were irradiated prior to infusion. Broad-spectrum antibiotics were administered for febrile neutropenic episodes. GGF was given on day +5 after allogeneic BMT. Patients who were serologically positive for CMV or who had donors who were serologically positive for CMV received ganciclovir prophylaxis after engraftment until day +100, and acyclovir thereafter. All patients received G-CSF 480 µg i.v. starting on day +1 until neutrophil engraftment.

All patients received the following prophylactic regimens for oral mucositis: Peridex mouthwash (15 cm³) t.i.d. and either mycostatin mouthwash t.i.d. or mycelex troche q.i.d. Engraftment was defined as follows: neutrophil engraftment was the day to an absolute neutrophil count of 500/µl on two consecutive days; platelet engraftment as the day to achieving a platelet count of 20,000/µl independent of platelet transfusions for 3 consecutive days.

Measurement of mucositis and GVHD

We used the modified OMAS mucositis tool to evaluate mucositis. The OMAS scale evaluates multiple regions of the oral cavity for erythema (none, mild, moderate, severe) and the presence of ulcerations or pseudomembranes. The scale ranges from 0 to 2.0; a score of 0.5 or greater is considered to be severe mucositis. Patients were evaluated for mucositis three times a week from admission to day +21. The severity of mucositis for an individual patient was defined as the maximum OMAS score recorded during the first 21 days post transplant. Acute GVHD was monitored weekly while patients were hospitalized for their allogeneic BMT, and at a minimum of weekly for the first 60 days, and a minimum of every 21 days for the first 100 days. Acute GVHD was graded using the NMDP scale for GVHD grading.

Statistical analysis

The primary end points for the study were incidence of acute GVHD and severe mucositis. We hypothesized that MMF would reduce the incidence of severe mucositis while not increasing the incidence of acute GVHD that is seen with MTX. The study was designed as a prospective, randomized study in which 80 patients were to be randomized 1:1 to receive MMF or MTX along with CSP for GVHD prophylaxis. The study was designed to detect an absolute difference of 35% in the incidence of severe mucositis between the two study arms (77% incidence with MTX, 47% with MMF) with 80% power using a two-sided significance level of 5%. No interim analyses were planned. However, due to slower than anticipated accrual, we decided to conduct an interim analysis after the first 40 patients were enrolled. The Lan and DeMets alpha-spending function with a Pocock stopping boundary was employed for this interim analysis. Using this approach, a nominal significance level of P<0.03 was needed for the results to be considered statistically significant. The major end points of the study were the incidence of severe mucositis and acute GVHD. The speed of hematopoietic engraftment was a secondary end point. If one arm of this trial resulted in a significantly more favorable result with respect to the incidence of severe mucositis or severe acute GVHD at the time of interim analysis, the study would be terminated. Categorical variables were summarized as frequencies and percentages, and compared between groups using the χ² test. Continuous variables were summarized as the median and range, and compared between groups using the Wilcoxon rank sum test. Survival, relapse, and relapse-free survival were calculated from the date of transplant. These outcomes were estimated using the Kaplan-Meier method and compared between groups using the log-rank test. All analyses were conducted using SAS® software (SAS Institute, Cary, NC, USA), and all statistical tests were two sided.

Results

In total, 21 patients received MMF and 19 patients received MTX. Patient demographics are shown in Table 1. The median follow-up of patients in the MMF arm is 23 months and 21 months in the MTX arm. The median dose of transplanted hematopoietic stem cells the patients received was similar in both groups: MMF patients received 2.53 x 10⁶ nucleated cells/kg (range 1.22-4.56) and MTX patients received 2.91 x 10⁶ nucleated cells/kg (range 1.06-5.45), (P=0.50). Bone marrow CD34+ cell dose was similar in both groups: MMF patients received 2.10 x 10⁶ CD34+ cells/kg (range 0.76-5.14); MTX patients received 2.43 x 10⁶ CD34+ cells/kg (range 0.76-5.34), (P=0.20). The degree of mucositis was significantly less in the MMF group. Median OMAS score was 0.25 (range 0-1.25) in the MMF group vs 1.0 (range 0-2.0) in the MTX group.

Bone Marrow Transplantation
Table 1  Patient demographics

| Variable | MMF  
| (n = 21) | MTX  
| (n = 19) |
|----------|------|------|
| Primary diagnosis | | |
| AML      | 16 (76%) | 13 (68%) |
| NHL      | 4 (19%)  | 1 (5%)  |
| MDS      | 1 (5%)   | 1 (5%)  |
| CMML     | 0       | 2 (10%) |
| CLL      | 0       | 1 (5%)  |
| HD       | 0       | 1 (5%)  |
| Risk category | | |
| Good     | 8 (38%) | 8 (42%) |
| Poor     | 13 (62%)| 11 (58%)|
| Median age at transplant (years) | 49 (19-60) | 46 (16-62) |
| Median months from diagnosis to transplant | 4.5 (1.8-37.2) | 4.1 (0.2-144.9) |

Figure 1  Mucositis post transplant: MMF vs MTX.

(P = 0.004) as shown in Figure 1. In addition, the MMF group had significantly less severe mucositis than the MTX group (21 vs 65%, P = 0.008). More patients in the MTX arm required narcotics to control pain associated with mucositis (94 vs 75%), and took narcotics for more days (15.5 vs 9.5) during the peri-transplant period. Additionally, 64% of patients in the MTX arm required TPN during the period of mucositis vs only 19% in the MMF arm.

Engraftment was faster in the MMF group. Median time until neutrophil engraftment was 11 days (range 8-24) with MMF vs 18 days (range 11-28) with MTX (P < 0.001). The median time until platelet engraftment was 19 days (range 13-44) in the MMF group vs 23 days (range 14-63) in the MTX group (P = 0.008). The combination of a more rapid engraftment and less severe mucositis leads to a shorter inpatient length of stay for the MMF group (median 27 vs 36 days, P < 0.001).

The incidence and severity of acute GVHD was similar in the two groups. The incidence of grade 0-1 acute GVHD was similar in the two arms (22% in the MMF arm and 26% in the MTX arm). The incidence of grade II-IV acute GVHD was 48% in the MMF arm and 37% in the MTX group (P = 0.49). The incidence of chronic GVHD was similar in both groups. For those who survived more than 100 days, the incidence of chronic GVHD was 63% in the MMF group and 64% in the MTX group (P = NS).

Interim analysis of the first 40 patients revealed that the use of CSP and MMF was associated with a reduced severity of mucositis, faster hematopoietic engraftment, similar incidence of acute GVHD events, and comparable survival (which resulted in premature study closure).

There was no significant difference in survival. The 6-month overall survival was 52% for the MMF group and 68% for the MTX group (P = 0.23); 6-month relapse-free survival was 48% (MMF) vs 53% (MTX), P = 0.53. There was no difference in rates of relapse between the two groups (38% MMF vs 47% MTX, P = 0.81, log-rank test). There was no difference in survival between the two groups when analyzing the good and poor risk groups separately.

In this high-risk group of patients, disease relapse was the most common cause of death, accounting for 47% of deaths in the MMF group and 50% of deaths in the MTX group. Other causes of death included acute GVHD (n = 2), chronic GVHD (n = 4), multisystem organ failure (n = 4), and other (n = 3).

The incidence of infections was similar in the two study groups. The incidence of CMV viremia post transplant was 43% in the MMF group and 47% in the MTX group. In total, 14 patients developed Gram negative bacteremia, seven in each study group. The most common organisms were pseudomonas aeruginosa (n = 4) and klebsiella pneumoniae (n = 3).

Discussion

It is well known that clinical GVHD is associated with a beneficial graft-versus-tumor effect. Unfortunately, excessive GVHD increases the morbidity and mortality associated with allogeneic BMT. Ideally, patients fare best who have mild to moderate GVHD without the toxicities of severe GVHD. Given the potential benefit of GVHD, it is not unreasonable to ask why any prophylaxis is used in HLA matched sibling transplantation in the first place. The reason is that in the absence of prophylaxis, the incidence of ‘hyperacute’ acute GVHD approaches 100% and is associated with overwhelming morbidity. Many strategies of GVHD prophylaxis have been explored, but the most common is the combination of CSP with MTX given on days 1, 3, 6, and 11 after allogeneic transplant. The combination appears to be superior than either agent used alone. A recent review from the EBMT showed that 93% of BMT centers used a combination of CSP and MTX for GVHD prophylaxis. While the initial dose of CSP is relatively uniform, aiming to achieve therapeutic blood levels, the dose of MTX varies, with doses ranging from 5 to 15 mg/m² used. Some centers also use a different schedule, dosing on days +2, +4, +8, and potentially +12. Finally, some centers utilize folic acid rescue after MTX.

Unfortunately, the use of a cytotoxic agent such as MTX has the potential to result in delayed engraftment. Additionally, MTX is frequently accompanied by mucositis, which may be quite severe. As a result, some centers omit the day +11 dose of MTX. One center has reported...
that the omission of the day +11 dose did not result in any negative long-term sequelae, but this report was contradicted by a subsequent report from another center.

MMF is a powerful immunosuppressive agent that inhibits the proliferation of T and B-lymphocytes. It has been used successfully to prevent and treat rejection in solid organ transplantation. Reports with limited patient numbers have shown efficacy of MMF, either alone or in combination with other agents, for GVHD prophylaxis as well as for the treatment of acute and chronic GVHD. Additionally, there appears to be synergism between MMF and CSP in preventing GVHD in dog models of transplantation. This had led to the well-reported use of the combination of CSP and MMF for the prevention of graft rejection, as well as for the prophylaxis of GVHD in nonmyeloablative allogeneic transplants.

In this setting, MMF is tolerated very well and this has allowed the nonmyeloablative transplant patients to frequently receive their care as outpatients.

The issue of mucositis in BMT is not trivial. It has been reported to contribute to a higher incidence of infection, and a longer inpatient length of stay in BMT patients. Increased regimen-related toxicity may be especially harmful to heavily pre-treated patients with refractory disease. Those patients frequently begin their transplants with periods of prior prolonged pancytopenia, and the added toxicity of mucositis induced by MTX may lead to serious clinical consequences. Thus, a GVHD prophylaxis regimen that reduces acute morbidities and achieves similar efficacy to CSP and MTX is noteworthy. In this prospective randomized trial we showed that patients receiving the combination of CSP and MMF had less severe mucositis, more rapid engraftment, and a shorter hospitalization, without an increased risk of infection. These clinically relevant results make the combination of CSP and MMF attractive in ablative transplants.

The primary limitation of this trial is the small sample size, and these results need to be verified in larger prospective randomized trials. Additionally, we used a busulfan-based preparative regimen, and it remains to be determined whether similar results would be achieved in patients receiving total body irradiation. We also did not use the most common dosage of MTX for GVHD prophylaxis. Most institutions use 15 mg/m² on day +1, followed by 10 mg/m² on days 3, 6, and 11; we used 5 mg/m² as described. This reduced dosage has been used at our institution, and others, for years, specifically designed to reduce the incidence and severity of mucositis associated with MTX. However, confirming the results of this trial in patients treated with a more common MTX dosing regimen would be beneficial. Another potential problem with this trial is the fact that we used mucositis as a primary end point that resulted in premature study closure. As a result, the study is not powered to detect significant differences in overall survival. Additionally, the follow-up is relatively short, and cannot adequately address potential differences in chronic GVHD. Finally, all of our donors used bone marrow and not peripheral blood progenitor cells (PBPCs). More rapid engraftment seen with PBPCs has the potential to yield different results.

An effective GVHD regimen that leads to reduced mucositis, faster hematopoietic engraftment, and the ability to be used in patients with hepatic or renal impairment is desirable. Additional studies designed to determine the efficacy of CSP plus MMF in other populations of patients undergoing myeloablative allogeneic BMT are warranted.

References

3. Niederwieser D, Matris M, Shizuru JA et al. Low-dose total body irradiation (TBI) and fludarabine followed by hematopoietic cell transplantation (HCT) from HLA-matched or mismatched unrelated donors and postgrafting immunosuppression with cyclosporine and mycophenolate mofetil (MMF) can induce durable complete remission and sustained remission in patients with hematologic disease. Blood 2003; 101: 1620–1629.


Cyclosporine, methotrexate, and methylprednisolone compared with cyclosporine and methotrexate for the prevention of graft-versus-host disease in bone marrow transplantation from HLA-identical sibling donor: a prospective randomized study

Tapani Ruutu, Liisa Voin, Terttu Parkkali, Eeva Juvonen, and Erkki Elonen

The role of corticosteroids in the prophylaxis of graft-versus-host disease (GVHD) is not well established. We conducted a prospective, randomized, open-label, single-center study about the effect of adding methylprednisolone (MP) to the widely used prophylactic regimen consisting of cyclosporine A and methotrexate. A total of 108 consecutive patients treated with allogeneic bone marrow transplantation from an HLA-identical sibling donor for malignant blood disease were entered into the study; 53 patients were randomized to receive MP and 55 were randomized not to receive prophylactic MP. The dose of MP was 0.5 mg/kg on days 14 to 20, 1 mg/kg on days 21 to 34, 0.5 mg/kg on days 35 to 48, and thereafter the dose was slowly tapered and the administration discontinued on day 110. In the group given prophylactic MP, the incidence of acute GVHD was lower (19% vs 56%, P = .0001), there was a trend toward a lower incidence of chronic GVHD among low-risk patients (P = .06), and during the first 4 months the time spent at hospital was shorter and there were fewer infections. The total amount of MP given was similar in the study groups because of a higher incidence of acute GVHD and its treatment in the group of patients not given prophylactic MP. There were no significant differences between the study groups in relapse rate or survival. In conclusion, the addition of MP to the combination of cyclosporine and methotrexate markedly reduced the incidence of acute GVHD without causing untoward effects. The timing of corticosteroid administration is probably important for the efficacy. (Blood. 2000;96:2391-2398)

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Introduction

Graft-versus-host disease (GVHD) with its consequences is the most important complication of allogeneic bone marrow transplantation (BMT), and its prevention and treatment are crucial for the success of this form of treatment. Cyclosporine A and methotrexate have been most commonly used to prevent GVHD, and the combination of cyclosporine and a short course of methotrexate is the most widely used form of prophylaxis. Corticosteroids are the first-line treatment of acute GVHD, but their role in prophylaxis is not well established. Corticosteroids have been used for the prophylaxis of GVHD together with cyclosporine, methotrexate, cyclophosphamide, tacrolimus, antimetabolite, and monoclonal rFAB-combined antibodies in various combinations. Studies of the effect of the addition of corticosteroids to the combination of cyclosporine and a short course of methotrexate have generally shown no benefit. In a randomized study, Storb and coworkers found that the addition of methylprednisolone (MP) to the combination of cyclosporine and methotrexate increased the incidence of acute GVHD. In that study, the administration of MP was started on the day of transplantation and continued until day 35. If corticosteroid treatment was postponed until day 15, no increase of acute GVHD was seen. In another randomized study using a similar schedule, the addition of MP to the combination of cyclosporine and methotrexate had no significant effect on GVHD. We report here a randomized prospective study about the effect of adding MP to the combination of cyclosporine and methotrexate for the prophylaxis of GVHD in 108 recipients of an allograft from an HLA-identical sibling donor using a different administration schedule. In this study, the addition of MP to the prophylactic regimen resulted in a marked decrease in the incidence of acute GVHD.

Patients and methods

A total of 108 consecutive adult patients treated for malignant hematologic disorder with allogeneic BMT from an HLA-identical sibling donor at Helsinki University Central Hospital were entered into the study from January 1999 to January 1999. The characteristics of the patients and donors are shown in Table 1. There were no significant differences between the study groups. Histocompatibility typing of the donors and recipients was performed by serology.

As conditioning treatment for transplantation, the patients received cyclophosphamide in a dose of 60 mg/kg of body weight on 2 successive days and either total body irradiation (TBI) or busulfan. Fifty-three patients participated in a multicenter study of the Nordic BMT Group comparing TBI and busulfan in the conditioning for BMT. The low-risk patients not participating in the study were given TBI with one exception, in which busulfan was given because radiotherapy was not available within a reasonable time. The high-risk patients were given TBI or busulfan according to individual judgment, depending, for example, on previous radiotherapy and the availability of TBI. TBI was given in 6 2-Gy doses during 5 days, days -4 to 0 in relation to the transplant; 1 dose was given per day with the exception of 2 doses given on day 1. The total dose was 12 Gy (lungs shielded to 10 Gy), and the dose rate was 4 Gy per minute.

From the Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland.

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Table 1. Characteristics of patients and donors

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‡ indicates acute myeloid leukemia; CML, chronic myeloid leukemia; ALL, acute lymphatic leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphatic leukemia.

Busalifan was given in the dose of 1 mg/kg 4 times daily for 4 days, for a total dose of 16 mg/kg, on days -5 to -2, followed by cyclophosphamide in the above-mentioned doses on days -1 to -3.

All patients received a bone marrow graft. The mean number of nucleated cells in the graft was 3.0 x 10^10/kg of the recipient's body weight (median 3.0, range 1.9-4.4) in the group given MP and 3.0 x 10^10/kg (median 3.0, range 1.6-4.4) in the group not given MP for prophylaxis. The grafts were nonmanipulated with the exception of the removal of red cells and plasma in cases of ABO incompatibility.

Postgrafting immunosuppression consisted of cyclosporine and methotrexate with or without MP according to randomization. Cyclosporine treatment was initiated on the day before transplantation and given in the dose of 3 mg/kg per day as continuous intravenous infusion. This was continued until approximately 2 weeks post-transplantation, when the patient was able to take the drug by mouth. The oral dose was 3 mg/kg per day taken in 2 parts at 12-hour intervals. This dose was modified in cases of adverse effects and to keep the whole-blood cyclosporine concentration under the level of 200 μg/L. Cyclosporine administration was continued until 1 year post-transplantation and then tapered off in approximately 6 weeks.

Methotrexate was administered at the dose of 15 mg/m² of the body surface area intravenously on day 0 and 10 mg/m² on days 3, 6, and 11 after grafting. Six hours after each dose, the same-milligram dose of calcium folinate was given intravenously.

Fifty-three patients were randomized to receive and 55 not to receive MP. The randomization was carried out with closed envelopes in sets of 4, separately for patients ever and under the age of 35 years. The schedule of MP administration is shown in Table 2. The drug was given orally. Acute 14,15 and chronic 16-17 GVHD were graded according to previously published criteria. All cases of gastrointestinal GVHD were biopsy-proven. Liver GVHD was documented either by liver biopsy or by biopsy-proven gastrointestinal GVHD and simultaneous clinical and laboratory findings compatible with liver GVHD. Because our previous experience had shown that skin biopsies taken early at the onset of acute GVHD yielded too inconclusive results to be used as a basis of treatment decisions and because our policy to treat GVHD early and intensively hampered the use of later biopsies, skin GVHD was in most present cases diagnosed on clinical grounds. Acute GVHD was treated at its appearance, independent of the grade, with 10 mg/kg per day of MP divided into 4 doses intravenously. The dose was halved every 3 days thrice and then tapered according to the clinical situation. In corticosteroid-resistant cases, antilymphocyte globulin (Atgam, Unipha, Kalamazoo, MI) was used as second-line treatment. The treatment of chronic GVHD consisted of MP and, according to individual judgment, cyclosporine, thalidomide, psoralen plus ultraviolet A, and low-dose irradiation of lymph nodes.

Survival and relapse-free survival were calculated from transplantation to death from any cause and to relapse or death, respectively. Relapse of acute leukemia was defined as more than 5% blasts in an essentially normocellular marrow. The relapses of chronic myeloid leukemia (CML) included both hematologic and cytogenetic relapses. Disease progression after the achievement of minimal disease state of myeloma, lymphoma, or chronic lymphatic leukemia after transplantation was also included in the analysis of the risk of relapse. The differences between the groups in the total dose of MP given, cyclosporine concentrations, infections, hospitalization days, and transfused red cell and platelet (pooled random donor) units were tested with the Mann-Whitney U test.

Results

Engraftment

All patients showed engraftment, with the minimum requirement of reaching 1.0 x 10^6 neutrophils/L. Table 3 shows the recovery of the neutrophil and platelet counts in the study groups. One patient in each group received granulocyte colony-stimulating factor.

Table 2. Schedule of methylprednisolone administration

<table>
<thead>
<tr>
<th>Days post-transplantation</th>
<th>Dose, mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-20</td>
<td>0.5</td>
</tr>
<tr>
<td>21-34</td>
<td>1.0</td>
</tr>
<tr>
<td>35-48</td>
<td>0.5</td>
</tr>
<tr>
<td>49-69</td>
<td>0.25</td>
</tr>
<tr>
<td>70-89</td>
<td>0.12</td>
</tr>
<tr>
<td>90-99</td>
<td>0.12 every other day</td>
</tr>
<tr>
<td>100-110</td>
<td>0.05 every other day</td>
</tr>
</tbody>
</table>

Doses of 0.25 mg/kg/day or more were divided in 2 parts.
Table 3. Recovery of neutrophil and platelet counts, transfusions, and hospitalization after transplantation

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MP+ (n = 53)</th>
<th>MP− (n = 55)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to recovery, day post-transplantation</td>
<td>Median</td>
<td>Range</td>
<td>Median</td>
</tr>
<tr>
<td>Neutrophils &gt; 0.5 × 10⁹/L</td>
<td>17</td>
<td>12-30</td>
<td>20</td>
</tr>
<tr>
<td>Neutrophils &gt; 1.2 × 10⁹/L</td>
<td>19</td>
<td>13-36</td>
<td>24</td>
</tr>
<tr>
<td>Platelets &gt; 20 × 10⁹/L</td>
<td>17</td>
<td>12-29</td>
<td>16</td>
</tr>
<tr>
<td>Platelets &gt; 50 × 10⁹/L</td>
<td>20</td>
<td>14-341</td>
<td>21</td>
</tr>
<tr>
<td>Transfusions, units</td>
<td>First 4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>24</td>
<td>8-358</td>
<td>40</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>4</td>
<td>0-85</td>
<td>6</td>
</tr>
<tr>
<td>First year</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelets</td>
<td>28</td>
<td>8-358</td>
<td>48</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>4</td>
<td>0-86</td>
<td>8</td>
</tr>
<tr>
<td>Hospitalization, days</td>
<td>First 4 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>39</td>
<td>20-115</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>First year</td>
<td>53</td>
<td>20-174</td>
</tr>
</tbody>
</table>

Neutrophil recovery was significantly faster in the group given MP, and there was a trend toward faster platelet recovery.

Treatment

MP was given according to the prophylaxis schedule to all patients randomized to receive this drug. The day +11 dose of methotrexate was given to 7 patients randomized to get MP or to 17 patients randomized not to receive MP because of severe mucositis or liver toxicity. The cyclosporine blood concentrations in the 2 study groups were compared at 1, 2, 3, and 4 months. There were no significant differences between the groups at any time (figures not shown).

Acute GVHD

The cumulative incidence of acute GVHD and the maximum grades are shown in Figure 1 and Table 4. A total of 19% of the patients given MP for prophylaxis had acute GVHD compared with 36% of those not given MP. The difference in the cumulative incidence of acute GVHD is highly significant, $P = .0001$. In the groups given and not given MP, the proportions of patients with grade II-IV acute GVHD were 7 of 53 (13%) and 20 of 55 (36%), respectively ($P = .0005$), and those of patients with grade III-IV acute GVHD were 3 of 53 (6%) and 9 of 55 (16%), respectively ($P = .08$). Among the patients given prophylactic MP, there was a marginally significantly lower incidence of corticosteroid-resistant acute GVHD defined as needing antilymphocyte globulin for second-line treatment because of nonresponsiveness to MP (2 vs 8 patients, $P = .05$). Four of the 10 cases of acute GVHD in the group given MP became manifest late, between days 54 and 73, later than any case of GVHD in the control group. Because patients given more cytotoxic therapy might show more tissue toxicity and possibly have a higher risk of GVHD, low-risk patients (acute leukemia in first remission and CML in first chronic phase) were studied separately. The difference in the cumulative incidence of acute GVHD was similar to that between the entire randomization groups and was highly significant (Figure 2; Table 4). Fifteen percent of the low-risk patients randomized to receive and 57% of those randomized not to receive MP developed acute GVHD of any grade, and the respective proportions of patients with grade II-IV acute GVHD were 2 of 33 (6%) and 13 of 37 (35%).

![Graph showing cumulative incidence of grade II-IV acute GVHD in patients given or not given methylprednisolone](image)

**Figure 1. Cumulative Incidence of grade II-IV acute GVHD in patients given or not given MP.**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MP+</th>
<th>MP−</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute GVHD, all patients</td>
<td>10/53 (19%)</td>
<td>31/55 (56%)</td>
<td>.0001</td>
</tr>
<tr>
<td>Grade I</td>
<td>3</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Grade IV</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Grade II-IV</td>
<td>7 (13%)</td>
<td>20 (36%)</td>
<td>.005</td>
</tr>
<tr>
<td>Acute GVHD, low-risk patients</td>
<td>3/33 (9%)</td>
<td>21/37 (57%)</td>
<td>.0004</td>
</tr>
<tr>
<td>Grade I</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Grade II</td>
<td>1</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Grade III</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Grade IV</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Grade II-IV</td>
<td>2 (6%)</td>
<td>13 (35%)</td>
<td>.003</td>
</tr>
<tr>
<td>Chronic GVHD, all patients</td>
<td>18/50 (36%)</td>
<td>25/52 (48%)</td>
<td>.17</td>
</tr>
<tr>
<td>Limited</td>
<td>12</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Extensive</td>
<td>6</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Chronic GVHD, low-risk patients</td>
<td>10/33 (30%)</td>
<td>19/37 (51%)</td>
<td>.06</td>
</tr>
<tr>
<td>Limited</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Extensive</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*Significance of the difference in cumulative incidence.
†See Table 1.
‡At risk for at least 100 days.
Chronic GVHD

A total of 102 patients survived more than 100 days and hence were at risk for chronic GVHD. Thirty-six percent of the patients given MP and 48% of those not given MP for prophylaxis developed chronic GVHD (Figure 3; Table 4). The difference did not reach significance, but among the low-risk patients the difference in the incidence of chronic GVHD was almost significant (30% vs 51%, P = .06) (Figure 4; Table 4). There was no difference between the study groups in the distribution of the limited and extensive forms of chronic GVHD.

The amount of MP administered

Because there was considerably more acute GVHD in the group randomized not to be given prophylactic MP, more MP was used for the treatment in this group. The mean total dose of MP administered during the first 4 months post-transplantation for prophylaxis and treatment was 55 mg/kg (median 35 mg/kg, range 20-260 mg/kg) in the group given MP for prophylaxis and 64 mg/kg (median 74 mg/kg, range 0-224 mg/kg) in the group with no MP in the prophylactic regimen. The difference is not significant, but there was a trend toward more MP being given to the patients randomized not to receive prophylactic MP.

Relapse

Figures 5 and 6 show the cumulative incidence of relapse for all and low-risk patients. The relapses in the total patient material include disease progression after the achievement of minimal disease state of myeloma, lymphoma, or chronic lymphatic leukemia after transplantation. Of the patients given and of those not given prophylactic MP, 17 of 53 (32%) and 16 of 55 (29%) relapsed, respectively. Among the low-risk patients, the relapse rates were 9 of 33 (27%) in the group given and 7 of 37 (19%) in the group not given MP (nonsignificant). Three of the relapses were cytogenetic relapses of CML: 1 in the group with MP and 2 in the group without MP. Thus, there were no significant differences in the relapse rates between the study groups.

Infections

Table 5 shows the infections observed in the study groups during the first 4 months and the first year after transplantation. Cytomegalovirus (CMV) infection was diagnosed in case of CMV viremia (early antigen-positive) and symptoms or signs likely to be caused by this infection. In deep fungal infection there was histologic or microbiologic documentation of fungus from a deep organ or fungemia. Septicemia were microbiologically documented. Pneumonias included all lung infiltrates, regardless of etiology, except lung abnormalities seen simultaneously with documented sepsis, deep fungal infection, or CMV infection as defined above. Most viral infections other than CMV were herpes simplex infections.

During the first 4 months, there were significantly fewer pneumonias, other bacterial infections, and deep fungal infections among the patients given MP for prophylaxis. The difference in pneumonias remained highly significant for the first year after transplantation. There was an almost significant trend toward fewer
CMV infections in the patients randomized to receive MP. There was no difference in the incidence of sepsis. The total number of infection episodes was markedly lower in the group given MP.

**Avascular bone necrosis**

Magnetic resonance imaging was carried out in patients with symptoms suggestive of avascular bone necrosis. This complication was diagnosed in 6 patients in both groups. There was no difference in the time of appearance of the complication, the median time post-transplantation being 13 months (range 6-54 months) among the patients given and 21 months (range 2-110 months) among those not given prophylactic MP.

**Blood transfusions and hospitalization**

Post-transplantation blood cell transfusions during the first 4 months and the first year are shown in Table 3. There was an almost significant trend toward fewer blood cell units given in the MP+ randomization group.

The time spent at the hospital during the first 4 months was significantly shorter among the patients given MP for prophylaxis (Table 3).

**Survival**

Figures 7 and 8 show the survivals of all and low-risk patients according to the study arm. At the time of the analysis, 32 of the 53 patients (60%) given MP and 28 of the 55 (51%) not given MP were surviving. Among the low-risk patients, the proportions of surviving patients were 25 of 33 (76%) for the patients given and 25 of 37 (68%) for those not given MP. The survivals did not differ significantly.

**Causes of death**

The principal causes of death are shown in Table 6. Relapse was the most common cause of death, followed by GVHD. There was no difference between the study groups; neither was there any difference in the causes of death among the low-risk patients. Five low-risk patients died of relapse in the group given and 6 in the group not given MP. Two low-risk patients in the MP+ group and 4 in the MP− group died of GVHD.

**Discussion**

Corticosteroids are effective in the treatment of most patients with acute GVHD and should therefore also be useful in the prophylaxis of GVHD. However, their role in the prevention of GVHD is not well established. Including the present study, the addition of corticosteroid to the most widely used prophylactic regimen, the combination of cyclosporine and methotrexate, has been evaluated in 3 fully published randomized trials with contradictory results. In the study of Storb and coworkers, the addition of MP to cyclosporine and methotrexate increased the incidence of acute and chronic GVHD in sibling transplantations, whereas in the study of Atkinson et al with small numbers of patients, no difference in GVHD was seen between the patients given and those not given prednisolone. In the present study, the addition of MP to cyclosporine and methotrexate highly significantly reduced the incidence of acute GVHD. In the study group given MP, 4 of the 10 cases of acute GVHD became manifest late, during the tapering of the MP dose. Late occurrence of acute GVHD among patients given corticosteroid was also observed in the studies by Storb et al and Atkinson et al.

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**Table 5. Infections in patients given or not given methylprednisolone**

<table>
<thead>
<tr>
<th>Type of Infection</th>
<th>First 4 months (patients/episodes)</th>
<th>First year (patients/episodes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MP+ (n = 53)</td>
<td>MP− (n = 55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepsis</td>
<td>13/15</td>
<td>12/12</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>4/4</td>
<td>13/16</td>
</tr>
<tr>
<td>Other bacterial</td>
<td>10/12</td>
<td>20/24</td>
</tr>
<tr>
<td>Deep fungal</td>
<td>0/0</td>
<td>5/5</td>
</tr>
<tr>
<td>CMV</td>
<td>9/9</td>
<td>18/20</td>
</tr>
<tr>
<td>Other virus</td>
<td>12/13</td>
<td>14/23</td>
</tr>
<tr>
<td>All infection</td>
<td>53</td>
<td>59</td>
</tr>
</tbody>
</table>

*Difference between the numbers of patients with infection.
Another study showing no benefit of the addition of prednisone to the combination of cyclosporine and methotrexate has recently been published in the abstract form,\textsuperscript{18} but because details of the treatment, such as drug dosages and timing of administration were not given, this study cannot be further discussed here.

There were differences in the prophylactic scheme between the 2 previous studies and the present one, which are likely to explain the differences in the results. The most obvious difference is the timing of corticosteroid administration. In the previous studies, the corticosteroid treatment was initiated on the day of transplantation at a dose of 1 mg/kg per day; the dose was halved at 3 weeks and discontinued 30 to 35 days post-transplantation. In the present study, MP was started on day 14 after transplantation at 0.5 mg/kg per day, the maximum dose of 1 mg/kg per day was given on days 21 to 35, and thereafter the dose was slowly tapered and the administration discontinued on day 110. The reasoning behind this schedule was 2-fold: MP was initiated only after methotrexate administration was completed to avoid any interference with the effect of this drug, and the highest dose was scheduled to be given at the time of the highest risk of the appearance of symptomatic GVHD. In our previous experience, the median time of the appearance of acute GVHD in patients given cyclosporine and methotrexate prophylaxis was day 26 post-transplantation. The importance of the timing of corticosteroid administration is supported by the observation in the Seattle study\textsuperscript{16} that if the initiation of MP treatment was postponed to day 15, the increasing effect on the incidence of acute GVHD disappeared.

Another difference between the present study and the 2 previous ones is the dosing of cyclosporine. The intravenous dose at the early stage was the same, but after the switch to oral route we gave a much lower dose: 3 mg/kg per day compared with 12.5 mg/kg per day. We chose this low dose because preliminary information indicated that patients on low-dose cyclosporine may do at least as well as those on a higher dose and that increasing the intensity of GVHD prophylaxis may increase the relapse rate.\textsuperscript{19} We also had significant problems with thrombotic microangiopathy at the time of designing the present study. Lower cyclosporine doses might have increased the incidence of acute GVHD among the patients not given MP compared with the other studies. This does not, however, seem to be the case. Because we also treated grade I GVHD, which apparently differs from the policy applied in other studies, the comparison is not perfectly valid, but the incidence and severity of acute GVHD do not seem to be essentially different in the 3 studies. The incidence of grade II-IV acute GVHD among patients not given corticosteroid prophylaxis was 36% in both the Seattle study\textsuperscript{16} and the present one. There was less grade II-IV acute GVHD in the monocorticosteroid group of Atkinson et al\textsuperscript{11} than in the present study (15% vs 36%) but more grade I-IV GVHD (75% vs 56%). These differences may be partly explained by the often vague separation of grade I from grade II in clinical practice. Thus, the beneficial effect of the addition of MP in the present study does not appear to have been caused by an unduly high GVHD incidence in the control arm.

A further detail where the 3 studies may have differed is the use of folic acid rescue after methotrexate treatment, as in the present study. This detail is not usually reported but, according to a recent survey among European centers, approximately one third of the centers gave folic acid subsequent to methotrexate administration.\textsuperscript{1}

More patients in the group not given MP had day 111 methotrexate omitted because of toxicity—i.e., most cases severe mucositis—compared with the triple prophylaxis group. There were no obvious differences in the baseline characteristics of the study groups to explain the difference in toxicity, which might have been a mere chance occurrence. The difference in the methotrexate administration could have been one reason for the difference in the incidence of acute GVHD. However, there was a trend toward less acute GVHD among the patients not given the fourth dose of methotrexate compared with those given the full course; the incidence was 14% versus 21% in the group given MP and 41% versus 65% in the group not given MP, respectively. Therefore, the fact that fewer patients in the group not given prophylactic MP received the fourth dose of methotrexate does not explain the higher incidence of acute GVHD in this group.

A trend was observed toward less chronic GVHD in the group given prophylactic MP. Although this effect did not reach significance, the trend may indicate that a low dose of corticosteroids during the first 3 to 4 months after transplantation may have some prophylactic effect on chronic GVHD. This is logical because corticosteroid is the first-line treatment of chronic GVHD. Storb and coworkers\textsuperscript{10} observed an increase in chronic GVHD among patients administered prophylaxis with MP in addition to cyclosporine and methotrexate compared with those not given MP. Likewise, Deeg et al\textsuperscript{1} found that the addition of MP to cyclosporine prophylaxis increased the incidence of chronic GVHD. The causes of these unexpected findings and the difference from the results of the present study are not clear, but an obvious factor is the duration of MP prophylaxis. We gave MP until day 110 post-transplantation, whereas the administration was discontinued on day 35 in the study by Storb et al\textsuperscript{10} and on day 72 in the study by Deeg et al.\textsuperscript{3}

In some studies, the intensification of GVHD prophylaxis has been associated with an increased risk of relapse.\textsuperscript{20,21} In the present study, the addition of MP to the prophylactic regimen had no effect on the relapse rate. It may be noteworthy that we used relatively low cyclosporine doses.

Table 6. Principal causes of death

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MP+(n = 53)</th>
<th>MP-(n = 55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapse</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>GVHD</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Infection</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Intracerebral hemorrhage</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ARDS, DIC</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>27</td>
</tr>
</tbody>
</table>

ARDS indicates acute respiratory distress syndrome; DIC, disseminated intravascular coagulation.
There were fewer infections among the patients given MP for prophylaxis than in the control group. Because GVHD, especially chronic GVHD, is associated with immunosuppression, the low incidence of GVHD among the patients given prophylactic MP may at least partly explain the lower infection rate. Because pneumonia after BMT is often associated with immunologic processes, the reduction of GVHD may explain the lower rate of pneumonias. High doses of MP, more often used for the treatment of acute GVHD in the study group not given prophylactic corticosteroid, probably contributed to the higher incidence of infections.

The recovery of the neutrophil counts after transplantation was significantly faster among the patients who received MP prophylaxis, as also reported previously by Storb et al.10 This was seen despite the fact that more patients not given MP for prophylaxis had the last methotrexate dose omitted because of toxicity. The faster neutrophil recovery was probably caused by demargination because there was no significant difference in the platelet recovery. The nonsignificant trend toward fewer blood cell transfusions given to patients with MP prophylaxis may reflect the lower incidence of GVHD in this study group. GVHD is associated with cytopenias in a significant proportion of patients; the mechanisms may be manifold and complex.24,25 The significantly shorter hospitalization during the first 4 months in the group randomized to receive prophylactic MP was due to the lower incidence of acute GVHD and fewer infections.

The adverse effects of corticosteroids, particularly infections, hypertension, hyperglycemia, and avascular bone necrosis are well known, and they have to be taken into account when weighing the advantages and disadvantages of corticosteroid administration. In the present study, the prophylactic use of MP did not result in greater exposure to corticosteroids because there was markedly more acute GVHD in the control group and the doses used for treatment were higher than the prophylactic doses. As shown above, there were fewer infections in the group given MP prophylaxis. Blood pressure and glucose balance were not prospectively recorded. There were no differences in the incidence or time of the onset of avascular bone necrosis.

The treatment policy of acute GVHD applied in this study was aggressive. We treated even early (grade 1) acute GVHD with high-dose corticosteroids to stop effectively the GVHD process. While this policy may be more efficient in the treatment of acute GVHD than a more conservative approach, adverse effects may outweigh the benefits. In a registry study of the European Group for Blood and Marrow Transplantation (EBMT), it was found that patients treated for acute GVHD at centers applying a very intense treatment policy had a worse outcome than those treated at centers applying a more conservative approach.26 The treatment of all grades, including grade 1, at first signs with initially at least 10 mg/kg per day of MP resulted in poorer survival and higher mortality in infections and poor graft function than the treatment of only grade II+ acute GVHD with 2 mg/kg per day or less. Although these results were only suggestive due to the nature of the study, the findings indicated that a very intense treatment policy may not be optimal for the outcome. In the present study, the patients randomized not to receive MP did, in fact, receive as much MP on the average as those randomized to receive MP for prophylaxis because of markedly more GVHD and intense treatment.

The randomization was not stratified according to risk groups. Therefore, the analysis of the outcome of low-risk patients is a retrospective subgroup analysis. However, the distribution of low-risk patients in the randomization groups was balanced, and there were no significant differences between the low-risk groups. The analysis of the outcome parameters of these more homogeneous patient groups showed findings in line with the results obtained in the analysis of the entire treatment groups. It was especially useful to observe that there was no difference in the relapse rate among the low-risk patients given or not given MP for prophylaxis.

In conclusion, the present study showed that the addition of MP to the combination of cyclosporine and methotrexate markedly reduced the incidence of acute GVHD without causing untoward effects. Based on the results of 2 previous studies and the present one, it appears that the timing of corticosteroid administration is important for the effect. There was a trend toward less chronic GVHD among the patients administered prophylactic MP. The addition of MP had no effect on the relapse rate or survival.

References

Low-dose cyclosporine of short duration increases the risk of mild and moderate GVHD and reduces the risk of relapse in HLA-identical sibling marrow transplant recipients with leukaemia

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Summary:

Low-dose cyclosporine (CsA), starting at 1 mg/kg/day i.v. with early discontinuation, and four doses of methotrexate (MTX), was given to 82 consecutive leukaemic patients receiving HLA-identical sibling marrow transplants. Retrospective controls (n = 40) received CsA, starting at 5–7.5 mg/kg/day i.v., given for 1 year, and MTX. In the low-dose group, the risk of acute GVHD grades I–II was 78% as compared to 57% among the controls (P < 0.01). The risk of acute GVHD grades III–IV was 2% and 5%, respectively (NS). Chronic GVHD occurred in 60% in the low-dose group and 24% in the controls (P < 0.001). Extensive chronic GVHD did not differ between the groups (3% vs 6%). In multivariate analyses, low-dose CsA was the only factor associated with acute GVHD grades I–IV (P = 0.02). Significant risk factors for chronic GVHD included low-dose CsA (P = 0.002) and CML (P = 0.03). Transplant-related mortality at 3 years post-BMT was 22% and 19%, in the low-dose group and controls, respectively (NS). The probability of relapse was 26% in the low-dose group and 53% in the controls (P = 0.06). In multivariate analysis, high-dose CsA was the strongest risk factor for relapse (P = 0.03). The 3-year relapse-free survival was 58% in the low-dose group and 43% in the controls (P = 0.1).

Keywords: relapse; immunosuppression; graft-versus-host disease; bone marrow transplantation

Cyclosporine A (CsA), together with a short course of methotrexate (MTX), is today the most commonly used protocol for the prevention of graft-versus-host disease (GVHD) after allogeneic BMT.1–4 The potent immunosuppressive effect and the pronounced side-effects of CsA have been described elsewhere in detail.5–7 CsA has often been given i.v. from the day before BMT in doses ranging from 3 to 5 mg/kg/day. However, up to 10–20 mg/kg/day has also been used in the earlier era of BMT.1,2,8 The schedules for administration vary between centres and have also been modified over time. Previous studies have shown that the use of high doses of CsA, to minimise the incidence of severe GVHD, not only correlates with increased organ toxicity, but also leukaemic relapse.9 This is believed to be caused by a diminished graft-versus-leukaemia effect (GVL).10–13 Low-dose CsA was also reported to reduce the relapse incidence and increase patient survival.9 Several methods are available for monitoring serum and blood levels of CsA.7 These levels have been reported by some investigators to correlate with the grade of GVHD,14 although others have shown no correlation.15,16 This discrepancy can be explained to some extent by a large inter-individual variation in bioavailability.7 The optimal dosage and duration of CsA treatment have yet to be found. Customarily, many centres have given CsA for 6 months post-BMT. Some studies have reported safe discontinuation of CsA at day 60 or 90 after BMT.14,17 In contrast, a study from our centre showed an increased incidence of chronic GVHD if CsA was discontinued after a median of 71 days, as compared to 1 year.18

In the present study, we aimed to compare the clinical outcome in leukaemic patients receiving low-dose CsA (starting at 1 mg/kg/day i.v.) aiming at 2 months’ duration with retrospective controls receiving CsA, starting at 5–7.5 mg/kg/day i.v. and given for 1 year. The CsA doses and serum levels during the first 3 weeks after BMT have been reported to be crucial to the development of GVHD and the GVL effect.9 Therefore we aimed to compare blood levels of CsA during this time period and analyse these in relation to the actual dose of CsA given and clinical outcome. We also wished to assess the impact of low-dose CsA on the incidence of GVHD and relapse in the presence of other, previously reported, potential risk factors.19–21

Materials and methods

Patients and donors

Eighty-two patients with leukaemia (AML, ALL and CML), receiving allogeneic stem cell grafts between February 1993 and June 1998, were included in the study group, all of them having HLA-A, -B and DR identical sibling donors and receiving the low-dose CsA protocol (starting at 1 mg/kg/day i.v., described below), in combination with MTX. This group was compared with retrospective controls (n = 40), transplanted between 1985 and 1993, and receiving CsA starting at 5–7.5 mg/kg/day i.v., in combination...
with MTX. The main clinical data of the patients (study group and controls) and their donors are depicted in Table 1.

**Conditioning**

The majority of patients (*n* = 111) were conditioned with CY 120 mg/kg and TBI 10 Gy in one setting, as previously described. Eleven patients were conditioned with a small BUCY regimen, consisting of BU 4 mg/kg p.o. in divided doses daily for 4 days, in combination with 120 mg/kg of CY (10 patients in the low-dose group and one patient among the controls). Before November 1988, all patients received 8-12 mg MTX or 20 mg Ara-C intrathecally (IT) twice before BMT to prevent CNS leukaemia. Post-transplant, IT MTX was given from day 32 and every other week until day 102. After 1988, only patients with ALL, AML M4 and M5 and/or with a history of CNS disease were given this treatment. To patients with previous CNS disease, IT MTX was thereafter given every 8th week until 24 months post-BMT.

**Immunosuppression and monitoring of CsA levels**

All patients received CsA in combination with four doses of MTX i.v., 15 mg/m² on day 1 and 10 mg/m² on days 3, 6 and 11. CsA treatment was initiated i.v. on the day before BMT. Patients receiving low-dose CsA were given 1 mg/kg/day i.v. divided into two or three doses from day -1. On day 1, or as soon as the patient could take CsA orally, 3 mg/kg/day was given divided into two doses. From 2 months after BMT, in the absence of GVHD, the CsA dose was gradually tapered. Among the retrospective controls, CsA treatment was started at 5-7.5 mg/kg/day. From day 1, or as soon as possible, CsA was given orally 12.5 mg/kg/day, and the highest tolerated CsA dose, with respect to serum creatinine levels, was given for 6 months. The dose was then gradually tapered and, in the absence of GVHD, discontinued after 1 year. In the low-dose group, CsA doses were adjusted according to blood levels and/or if toxicity was seen. We aimed at maintaining the CsA blood levels around 100 ng/ml. Three different methods for determining CsA blood levels have been used from 1985 up until today, without altering the reference values. Between 1985 and April 1988, the Sandimmun kit (formerly manufactured by Sandox, Basel, Switzerland) was used. From April 1988 until May 1997, we used the Cyclo-trac SP® (Inestar, Stillwater, MN, USA). Both of these methods were specific radioimmunoassays. Since May 1997, we use the EMIT 2000 Cyclosporine-specific Assay (Behring, Cupertino, CA, USA).

**Grading and treatment of GVHD**

Acute GVHD was graded from 0 to IV according to Glucksberg et al. Grade I acute GVHD was treated with 2 mg/kg/day of prednisolone for 1 week and the dose was then tapered. In patients with progressive symptoms, prednisolone was continued for a longer period. In some severe cases, methylprednisolone (0.125–0.5 g/day), ATG, additional MTX or PUVA were also given. Chronic GVHD was graded as limited or extensive. It was initially treated with CsA and steroids. If a response was not seen, some patients received TLI, PUVA, and more recently extracorporeal PUVA.

**Statistical analysis**

The results were analysed as of 15 October 1998, allowing for a median follow-up time of 38 months (range 5-68 months) in the low-dose group. Time to GVHD, relapse, survival, etc., were analysed by the life-table method with the log-rank test (Mantel-Haenszel), taking censored data into account. Non-parametric analysis was performed with the Wilcoxon’s signed rank test and the Mann-Whitney U test was used for paired and unpaired data, respectively. When analysing the risk of relapse and leukaemia-free survival (LFS), a separate subgroup analysis for patients in early and advanced disease was also performed. When evaluating risk factors for acute GVHD, the logistic regression model was used in uni- and multivariate analyses. All patients with a survival of at least 30 days were analysed and grades I-IV were regarded as the event. We evaluated the low-dose regimen among eight other risk factors, previously reported to be significant at our centre. These included donor and recipient age, sex-match (including immunised female-to-male recipient), time to engraftment and pretransplant herpes virus serology for CMV, HSV, VZV and EBV (Table 2). When analysing risk factors for chronic GVHD, relapse, and leukaemia-free survival, the Cox regression model was used for the uni- and multivariate analyses. Nineteen risk factors for chronic GVHD and six risk factors for relapse, previously reported to be significant in univariate analysis from our centre, were evaluated together with the low-dose regimen respectively (see Tables 3 and 4). When evaluating age as a risk factor for chronic GVHD a continuous variable was used. This is in contrast with the analysis of risk factors for acute GVHD where an age cut-off at 17 years was used.

### Table 1: Patient and donor characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low-dose CsA</th>
<th>Controls</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>82</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>33 (40%)</td>
<td>14 (35%)</td>
<td>NS</td>
</tr>
<tr>
<td>ALL</td>
<td>19 (23%)</td>
<td>11 (28%)</td>
<td>NS</td>
</tr>
<tr>
<td>CML</td>
<td>30 (37%)</td>
<td>15 (37%)</td>
<td>NS</td>
</tr>
<tr>
<td>First CR or CP</td>
<td>56 (68%)</td>
<td>24 (60%)</td>
<td>NS</td>
</tr>
<tr>
<td>Donor age</td>
<td>37 (2-61)</td>
<td>26 (3-59)</td>
<td>0.07</td>
</tr>
<tr>
<td>Donor sex (M/F)</td>
<td>42/40</td>
<td>20/20</td>
<td></td>
</tr>
<tr>
<td>Recipient age</td>
<td>38 (1-58)</td>
<td>32 (1-52)</td>
<td>NS</td>
</tr>
<tr>
<td>Recipient sex (M/F)</td>
<td>30/32</td>
<td>25/15</td>
<td></td>
</tr>
<tr>
<td>CMV seronegative donor and recipient</td>
<td>8 (10%)</td>
<td>8 (10%)</td>
<td>NS</td>
</tr>
<tr>
<td>Non immunised F to M*</td>
<td>11 (13%)</td>
<td>6 (13%)</td>
<td>NS</td>
</tr>
<tr>
<td>Immunised F to M*</td>
<td>13 (16%)</td>
<td>5 (13%)</td>
<td>NS</td>
</tr>
<tr>
<td>Cell dose infused*</td>
<td>2.5 (1.1-10.7)</td>
<td>2.2 (1.0-9.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

*By pregnancy or previous blood transfusion.
*Nucleated cells x 10⁹/kg.
CR = complete remission; CP = chronic phase; M = male; F = female; F to M = female donor to male recipient; NS = not significant (*p > 0.05).
Table 2: Risk factors for acute GVHD, univariate analysis, logistic regression

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of patients</th>
<th>Relative hazard</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age &lt;17 or 17 years</td>
<td>21/101</td>
<td>1.52</td>
<td>0.24</td>
</tr>
<tr>
<td>Donor age &lt;17 or 17 years</td>
<td>27/95</td>
<td>2.33</td>
<td>0.08</td>
</tr>
<tr>
<td>Female donor to male recipient (yes/no)</td>
<td>87/35</td>
<td>0.72</td>
<td>0.46</td>
</tr>
<tr>
<td>Female immunised donor to male recipient (yes/no)</td>
<td>104/18</td>
<td>1.27</td>
<td>0.69</td>
</tr>
<tr>
<td>Recipient herpes virus serology (0-2/3-4)</td>
<td>19/96</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>Donor herpes virus serology (0-2/3-4)</td>
<td>25/87</td>
<td>0.79</td>
<td>0.64</td>
</tr>
<tr>
<td>Recipient CMV serology (neg/pos)</td>
<td>31/91</td>
<td>1.11</td>
<td>0.82</td>
</tr>
<tr>
<td>Donor CMV serology (neg/pos)</td>
<td>41/81</td>
<td>1.21</td>
<td>0.66</td>
</tr>
<tr>
<td>Engraftment (&lt;15 or 15 days)</td>
<td>58/54</td>
<td>0.72</td>
<td>0.43</td>
</tr>
<tr>
<td>High-dose CsA/low-dose CsA</td>
<td>40/92</td>
<td>2.61</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*Immunised by pregnancy or transfusion.
*White blood cell count >0.2 x 10^9/L.
Bold type indicates a P value <0.05.

Table 3: Cox univariate analysis of risk factors for chronic GVHD

<table>
<thead>
<tr>
<th>Factor</th>
<th>No. of patients</th>
<th>Relative hazard</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient age (continuous variable)</td>
<td>104/18</td>
<td>1.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Donor age (continuous variable)</td>
<td>97/73</td>
<td>1.02</td>
<td>0.97</td>
</tr>
<tr>
<td>Female immunised donor to male recipient</td>
<td>104/18</td>
<td>0.93</td>
<td>0.38</td>
</tr>
<tr>
<td>Recipient herpes virus serology (0-2/3-4)</td>
<td>19/96</td>
<td>0.97</td>
<td>0.95</td>
</tr>
<tr>
<td>Donor herpes virus serology (0-2/3-4)</td>
<td>25/87</td>
<td>1.58</td>
<td>0.23</td>
</tr>
<tr>
<td>Recipient CMV serology (neg/pos)</td>
<td>31/91</td>
<td>1.10</td>
<td>0.77</td>
</tr>
<tr>
<td>Donor CMV serology (neg/pos)</td>
<td>38/81</td>
<td>1.23</td>
<td>0.72</td>
</tr>
<tr>
<td>Donor and recipient CMV seropositivity</td>
<td>55/67</td>
<td>1.31</td>
<td>0.52</td>
</tr>
<tr>
<td>Recipient HSV serology (neg/pos)</td>
<td>25/96</td>
<td>0.79</td>
<td>0.57</td>
</tr>
<tr>
<td>Donor HSV serology (neg/pos)</td>
<td>49/73</td>
<td>1.28</td>
<td>0.41</td>
</tr>
<tr>
<td>Donor and recipient HSV seropositivity</td>
<td>59/65</td>
<td>1.46</td>
<td>0.20</td>
</tr>
<tr>
<td>Recipient VZV serology (neg/pos)</td>
<td>8/14</td>
<td>1.64</td>
<td>0.04</td>
</tr>
<tr>
<td>Donor VZV serology (neg/pos)</td>
<td>11/11</td>
<td>2.72</td>
<td>0.32</td>
</tr>
<tr>
<td>Acute myelopoiesis/CML</td>
<td>77/45</td>
<td>1.97</td>
<td>0.06</td>
</tr>
<tr>
<td>Marrow cell dose &lt;5 x 10^9/L (nucleated cells x 10^9/kg)</td>
<td>63/60</td>
<td>0.89</td>
<td>0.72</td>
</tr>
<tr>
<td>Acute GVHD 0-1 IV</td>
<td>33/87</td>
<td>2.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Acute GVHD 0-1 II-3 IV</td>
<td>108/12</td>
<td>1.31</td>
<td>0.61</td>
</tr>
<tr>
<td>HSVG infection (primary or reactivation)</td>
<td>92/29</td>
<td>0.92</td>
<td>0.81</td>
</tr>
<tr>
<td>Recipient lymphocyte transfusions (yes/no)</td>
<td>104/14</td>
<td>1.49</td>
<td>0.30</td>
</tr>
<tr>
<td>High-dose CsA/low-dose CsA</td>
<td>40/92</td>
<td>3.16</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Continuous variable 1-year increment.
*Immunised by pregnancy or transfusion.
**Buffy coat, booster marrow cells or T cells.
Bold type indicates a P value <0.05.

The reason for this discrepancy is that the age factor is presented in the way that it was originally used in our previous two studies. 26 For chronic GVHD, all patients surviving at least 90 days after BMT, and therefore at risk, were included in the analyses. Multivariate analysis of risk factors for relapse was performed both from day 90 and from the day of transplant.

Four risk factors, previously reported to correlate with inferior FS, 21 were evaluated together with the low-dose regimen. These included acute GVHD grades II-IV, chronic GVHD, disease stage, transplant herpes virus serology for CMV, HSV, VZV and EBV, and TBI as compared to BU.

All CsA concentrations for the first 3 weeks after BMT were collected from patient records. The CsA dose and patient weight given on day 22 were also recorded. When analysing CsA concentrations, we calculated the geometric means for each individual patient for the first 3 weeks post-BMT. These means were then added, creating arithmetic means, weekly and overall for the first 21 days.

Table 4: Multivariate analysis of risk factors for acute GVHD, chronic GVHD and relapse

<table>
<thead>
<tr>
<th>Factor</th>
<th>B</th>
<th>s.e.</th>
<th>RH</th>
<th>CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute GVHD</td>
<td>1.02</td>
<td>0.43</td>
<td>2.77</td>
<td>1.20-6.44</td>
<td>0.02</td>
</tr>
<tr>
<td>Low-dose CsA</td>
<td>1.21</td>
<td>0.29</td>
<td>3.35</td>
<td>1.56-7.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>0.66</td>
<td>0.30</td>
<td>1.94</td>
<td>1.08-3.48</td>
<td>0.03</td>
</tr>
<tr>
<td>Low-dose CsA</td>
<td>0.77</td>
<td>0.34</td>
<td>2.16</td>
<td>1.11-4.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>0.69</td>
<td>0.34</td>
<td>1.99</td>
<td>1.02-3.88</td>
<td>0.04</td>
</tr>
<tr>
<td>Relapse</td>
<td>0.80</td>
<td>0.41</td>
<td>2.23</td>
<td>1.00-4.97</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Logistic regression.
**Cox regression.
*As compared to first complete remission or chronic phase.
**B = β-coefficient; s.e. = standard error; RH = relative hazard; CI = confidence intervals (95%).
Bold type indicates a P value <0.05.

Table 5: Absolute incidences of acute and chronic GVHD

<table>
<thead>
<tr>
<th>Severity of GVHD</th>
<th>Low-dose</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>aGVHD: No.</td>
<td>17 (21%)</td>
<td>16 (41%)</td>
</tr>
<tr>
<td>I</td>
<td>54 (67%)</td>
<td>21 (34%)</td>
</tr>
<tr>
<td>II</td>
<td>8 (10%)</td>
<td>3 (5%)</td>
</tr>
<tr>
<td>III</td>
<td>2 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IV</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>GVHD: No.</td>
<td>38 (49%)</td>
<td>28 (78%)</td>
</tr>
<tr>
<td>Limited</td>
<td>37 (48%)</td>
<td>6 (17%)</td>
</tr>
<tr>
<td>Extensive</td>
<td>2 (3%)</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

*Acute GVHD: evaluable patients: 81 and 39, respectively.
**Chronic GVHD: evaluable patients: 77 and 36, respectively.

Results

CsA dose, serum levels and duration of treatment

In the low-dose group, patients had a median of seven CsA concentrations recorded during the first 3 weeks post-BMT (range 5-14). Among controls, the median number of CsA concentrations was 10 (range 0-17). In the low-dose group, where patients were to receive a p.o. dose of 3 mg/kg/day, the actual mean dose given on day 22 post-BMT was 4.6 mg/kg/day orally (range 0-11.8 mg/kg). Patients in the control group, who were to receive an initial p.o. dose of...
12.5 mg/kg/day, were actually given a mean dose of 12.7 mg/kg (range 0.250.250 mg/kg) (P < 0.001).

Mean blood levels in the low-dose group for weeks 1, 2 and 3 were 152, 156 and 162 ng/ml, respectively. In the controls with a long CsA course, the corresponding mean blood levels of CsA were 518, 543 and 605 ng/ml (P < 0.001).

Patients in the low-dose group had a median duration of CsA treatment of 6.6 months (range 2.5–42.6), compared to 11.7 (range 0.7–48.8) months in the control group (P = 0.01).

**Acute GVHD**

Patients receiving low-dose CsA ran an actuarial risk of acute GVHD grades I–II of 78%, compared to 57% in the control group (P < 0.01) (Figure 1). However, the incidences of acute GVHD grades III–IV were similar, 2% vs 5%, in the low-dose group and controls, respectively (NS) (Table 5).

In logistic regression using univariate analysis, low-dose CsA, as compared to high-dose, was the only significant risk factor for acute GVHD grades I–IV (P = 0.02) (Table 2). Donor age more than 17 years, as compared to younger donors, showed a trend towards significance (P = 0.08) and was therefore included in the subsequent multivariate analysis. However, only low-dose CsA maintained its significance in the multivariate analysis (P = 0.02) (Table 4).

**Chronic GVHD**

The actuarial risk of chronic GVHD was 60%, as compared to 24% in the controls (P < 0.001) (Figure 2). However, as depicted in Table 2, the risk of extensive chronic GVHD was the same in the two groups. In Cox regression univariate analysis, low-dose CsA (P < 0.01), recipient (P = 0.02) and donor age (P = 0.02) were significant factors associated with chronic GVHD. CML, as compared to acute leukaemia, and preceding acute GVHD (grades I–IV) were borderline significant (P = 0.06 and P = 0.06, respectively) and therefore were also included in the stepwise multivari-
Significant factors in multivariate analysis comprised high-dose CsA \( (P = 0.03) \), a more advanced disease \( (P = 0.04) \) and acute leukaemia \( (P = 0.05) \). Chronic GVHD did not maintain its significance in the multivariate analysis (data not shown). For this reason, multivariate analysis of risk factors for relapse, calculated from the day of transplant, is shown only (Table 4).

For 3-year LFS and actuarial patient survival the figures were 58% and 63% in the low-dose group, as compared to 43% and 50% among controls \( (P = 0.1 \) and \( P = 0.1 \), respectively) (Figure 4). Patients with early disease had an LFS of 63% in the low-dose group as compared to 42% in the control group \( (P = 0.6) \). For patients with advanced disease, the probabilities of LFS were 45% and 25%, in the low-dose group and the controls, respectively \( (P = 0.06) \). The only factor that was significantly associated with a superior LFS, in uni- and multivariate analysis was the presence of chronic GVHD \( (P = 0.02) \).

Discussion

Patients receiving GVHD prophylaxis with low-dose CsA ran a significantly higher risk of developing acute GVHD grades I-IV and less in those with a high control group. The mean dose of CsA given was 4.6 and 12.7 mg/kg/day orally in the two groups, respectively. In the low-dose group, we aimed to give 3 mg/kg/day orally. The increased dosage may be due to the fact that the CsA doses tended to increase in some patients developing acute GVHD. The median CsA level in blood was, therefore, also higher, 150 ng/ml, rather than 100, which was planned. CsA doses in the control group were given on the basis of the standard tolerable dose with respect to serum creatinine levels. One may therefore assume that this corresponds approximately to the mean CsA dose of 12.7 mg/kg/day seen in the control group. In a previous report by Bacigalupo et al., 81 allografted patients with acute leukaemia were randomized to receive CsA 1 or 5 mg/kg/day i.v. from day -1 to day +20. The actuarial risk of developing acute GVHD grades II-IV did not differ significantly between the groups in their study. However, in a Cox model, when stratifying for age and the CsA dose, the risk of acute GVHD grades II-IV was significantly higher among older patients (>20 years) and among patients receiving CsA 1 mg/kg/day. Multivariate analysis in our study showed that low-dose CsA was the only significant risk factor for developing acute GVHD grades I-IV. Unlike many other centres, we started to treat acute GVHD already at grade I. 14 This may partly explain why there were no differences in severe acute GVHD (grades III-IV) between our two groups.

Low-dose CsA was the strongest predictive factor for leukaemic relapse, when included in multivariate analysis \( (P = 0.03) \). These findings confirm those reported by Bacigalupo et al. 20 Concomitant treatment with cyclophosphamide and ciprofloxacin, during the pre-transplant conditioning, has been shown to correlate with an increased risk of relapse. 21 However, this risk factor was only present in the control group and could therefore not be evaluated separately in our analyses. Chronic GVHD was not significantly associated with an increased risk of relapse in multivariate analysis. One may speculate that when patients are given a significantly lower level of immunosuppression, the impact of chronic GVHD, as a risk factor for relapse, is of less importance. Interestingly, Bacigalupo et al. reported no difference in the risk of chronic GVHD between the high- and low-dose groups, in spite of a significant difference in relapse risk.

Figures for 3-year relapse-free survival and overall patient survival were in favour of our low-dose regimen, although differences between the groups did not reach significance. This may be due to the low number of patients. With more patients and a longer follow-up time, LFS may be significantly different, because TRM was the same in the two groups.

The duration of the CsA treatment was 6.6 and 11.7 months in the low-dose group and controls, respectively. We found that early discontinuation of CsA in the low-dose group was often complicated by ongoing mild acute GVHD, which prolonged the course of CsA. There have been reports where early discontinuation of CsA has not been associated with an increased risk of chronic GVHD. 16,17 In contrast, a previous study from our centre showed an increased incidence of chronic GVHD, if CsA was discontinued after a median of 71 days, instead of 1 year. 14 A recent study showed that preceding acute GVHD is often of less importance for the development of chronic GVHD than was found earlier. 30 This may be due to the use of MTX combined with CsA, in contrast to monotherapy. In line with this, early discontinuation of CsA contributed to an increased risk of developing chronic GVHD, as shown in our study (60% among low-dose patients vs 24% among controls), hence not merely an effect of low CsA dosage. The optimal LFS in leukaemic patients is seen in patients who develop grade 1 acute GVHD. 30 Since 21% of the patients did not develop acute GVHD at all, there may still be reason to reduce the CsA dose further, and/or discontinue CsA earlier in some patients.

We conclude from these data that a low-dose regimen with CsA, starting at a dose of 1 mg/kg/day i.v., in combination with four doses of methotrexate (MTX), is an advan-
tageous approach for recipients of HLA-identical sibling marrow transplants, with leukemia. The risk of mild acute GVHD and limited chronic GVHD increased in the low-dose group. This enhances the GVL effect, which subsequently reduces the risk of leukemic relapse post-BMT. This regimen appears safe because the risk of acute GVHD grades III-IV, TRM and extensive chronic GVHD did not increase.

Acknowledgements

We thank the Nursing Staff at the Allogeneic Stem Cell Transplantation Unit, Departments of Haematology and Paediatrics. The study was supported by grants from the Swedish Cancer Foundation (0070-B85-09XCC), the Children’s Cancer Foundation (1994-660), the Swedish Medical Research Council (B86- 16X-05971-16C), the Tobias Foundation, The FRF Foundation, Cancer and Trafikskadades Riksfo¨rbund and the Ellen Bachrach Foundation.

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27 Peto R, Pike MC, Armitage P et al. Design and analysis of


CHAPTER 5

CAMPATH MONOCLONAL ANTIBODIES

THE SECOND LOCAL INNOVATION
The Cambridge connection, as it has come to be widely known, proved to be a major research opportunity in the understanding and, equally important, management of immunologically mediated complications arising from allogeneic grafting\textsuperscript{150-152}. This was made possible through collaboration, which is actively ongoing, with Professor Herman Waldmann and Doctor Geoff Hale who have relocated their laboratory to Oxford\textsuperscript{153-155}. In the early studies it was demonstrated that reverse rejection or graft-versus-host disease in both acute and chronic forms could be markedly reduced by the presence of a specific immunoglobulin designed to blunt an initial phase of immune competence through paralysis of the T-helper lymphocyte population\textsuperscript{156-158}.

In parallel with the clinical studies being correlated, collectively analysed and then reported through the Campath users group that brought together investigators from many centres worldwide\textsuperscript{159-164} our early endeavours in the rabbit model, designed to further dissect this interrelationship\textsuperscript{162-164}, started to reveal an interesting new phenomenon.

Thus it was demonstrated that significant impact could be made on rejection. Not entirely surprisingly was the hypothesis that immune injury to recipient keratinocytes, biliary endothelium and enterocytes particularly of the small intestine that defined the acute graft-versus-host disease would turn out to be markedly reduced. These findings were first noticed with the initial IgM monoclonal antibody that was lytic and reduced visible numbers of lymphocytes. Comparably however was dramatically altered function with the next generation of IgG proteins that were opsonic also altering the course of this devastating adverse effect\textsuperscript{165-167}.

In a seminal laboratory study, widely accepted as landmark, we showed that, for the first time ever, an entirely different approach was possible and even more effective. Thus, by exposing the incoming graft \textit{ex vivo} or \textit{in vitro} to the same classes of this family of proteins\textsuperscript{20,168,169}, acute GVHD virtually disappeared.

Furthermore this pattern was preserved with the humanised variant designated as Campath 1H substantially limiting multiorgan lesions in the recipient with major outcome improvement. This research continues to be translated into treatment programmes in many countries\textsuperscript{170-172}.
Additionally systematic studies have, over many years, demonstrated that there is no associated increase in leukaemia relapse at least as far as acute myeloblastic subtypes are concerned\textsuperscript{173,174}.

Careful characterisation of the skin, liver and the bowel have led to the description of the new \textit{forme fruste} of acute immunologically mediated disease. This variant is readily responsive to topical corticosteroids and remarkable for having no consequential chronic expression. Meticulous characterisation of infectious complications have also failed to demonstrate any increase in cytomegalovirus infections.

These impressive outcome data continue to be reported on regular international basis\textsuperscript{155,159,168,171,175,176}. 
Campath-1 for prevention of graft-versus-host disease and graft rejection.
Summary of results from a multi-centre study

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The rat IgM antibody CAMPATH-1H recognizes an abundant family of glycoproteins
present on virtually all human lymphocytes and monocytes (1). We have obtained
rat IgG1, IgG2a, IgG2b and IgG2c antibodies with the same specificity and they are
lytic with human complement to a greater or lesser extent. Since 1982
CAMPATH-1H has been widely used for removal of T cells from human bone marrow to
prevent graft-versus-host disease (GVHD) (2). In 1986 we reported the combined
experience of the CAMPATH users group and noted a substantial reduction in the
incidence and severity of GVHD but an increased risk of graft rejection (3).
Other effective techniques for T cell depletion have all given similar results
(4). Subsequently there have been several suggestions of an increased relapse
risk following T cell depletion and this has been most clearly demonstrated in
CGL (5). Here we summarise the results of T cell depletion with CAMPATH-1H up to
December 1987 and outline a new approach to prevention of graft rejection using
the IgG2b CAMPATH-1G to give extra immunosuppression to transplant recipients.

A total of 441 matched sibling transplants for patients with leukaemia were
reported by 20 centres. There was no special selection of patients, and other
aspects of their treatment remained largely unchanged except that the majority
received no post-transplant immunosuppression and some patients received
additional total lymphoid irradiation (TLI) in an attempt to reduce the risk of
graft rejection. The median age of patients was 29 and the distribution by
disease was: ALL 98, AML 128, CGL 188, others 27.

T cell depletion of marrow buffy coat cells was accomplished by a single
TREATMENT with CAMPATH-1H and 25% autologous serum as described before (2). This
reduced the fraction of E-rosette forming cells to 1% or less in 90% of the
patients. Further depletion can be achieved by an extra round of complement
lysis, but this has not yet been used clinically. The median number of nucleated
cells infused was 2.3 x 10^9/kg. Engraftment was obtained in 393 patients with a
median time to reach 0.5 x 10^9 neutrophils/l of 19 days.

| Graft-versus-host disease and graft failure in matched sibling transplants |
|--------------------|----------|--------|--------|----------------|----------------|
|                    | No of  | Acute GVHD | Chronic GVHD |               |               |
|                    | patients| 0 1 2 3/4 | 0 M S | complete partial |
| GVHD prophylaxis: |         |         |         |                |                |
| 180 CyA, 3 Mtx     | 181     | 93 35 13 9 | 98 28 3 | 20 3           |
| none               | 260     | 127 26 14 18 | 128 13 4 | 44 4           |
| Total              | 441     | 220 61 27 27 | 226 41 7 | 64 7           |

Patients are scored 0 for GVHD only if they survived 100 days (acute) or 120 days
(chronic) with durable engraftment.
The incidence of acute GVHD was 18% grade 1, 8% grade 2 and 8% grade 3-4. The percentages are calculated as fraction of the 335 patients who survived to day 100 with durable engraftment or suffered from GVHD but died before day 100. Acute GVHD was not significantly affected by the use of prophylactic CyA but correlated with the infusion of more than 4 x 10^6 T cells/kg (P<0.0001). The incidence of chronic GVHD was particularly low; 15% mild/moderate and 3% severe (calculated as a percentage of 274 patients who survived to day 120 with durable engraftment). Chronic GVHD was not significantly associated with T cell dose but, paradoxically, it did correlate with the use of prophylactic CyA (P=0.008).

The incidence of graft failure was 15% complete and 3% partial (reversed by immunosuppressive treatment). It is likely that most graft failure was due to host rejection of the marrow. However, no particular conditioning regime seemed to be able to prevent graft rejection despite some attempts at intensification. The most promising method tested appeared to be the addition of TLI; however there were still 12 cases (11%) of graft failure among 108 patients who received TLI compared with 59 cases (18%) among 333 patients who did not receive TLI.

The risk of haematologic relapse was assessed for patients with acute leukaemia transplanted in first remission and for patients with chronic granulocytic leukaemia transplanted in chronic phase. So far the results for acute leukaemia do not differ significantly from published registry results for unpurged transplants, but the results in CGL illustrate the increase in relapse risk following T cell depletion.

### Actuarial probabilities of survival and of remaining in remission at 2 years

<table>
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<th>No of patients</th>
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<tr>
<td>ALL 1st remission</td>
<td>36</td>
<td>60 ± 10</td>
<td>80 ± 10</td>
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<tr>
<td>AML 1st remission</td>
<td>90</td>
<td>42 ± 8</td>
<td>74 ± 10</td>
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<td>CGL 1st chronic phase</td>
<td>142</td>
<td>69 ± 6</td>
<td>71 ± 7</td>
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<tr>
<td>with any GVHD</td>
<td>44</td>
<td>77 ± 8</td>
<td>86 ± 8 \ P=0.025</td>
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<tr>
<td>without GVHD</td>
<td>83</td>
<td>73 ± 7</td>
<td>63 ± 9 \ P=0.046</td>
</tr>
<tr>
<td>engraft &lt; 26 days</td>
<td>98</td>
<td>74 ± 6</td>
<td>78 ± 7 \ P=0.046</td>
</tr>
<tr>
<td>engraft &gt; 25 days</td>
<td>29</td>
<td>75 ± 13</td>
<td>52 ± 18</td>
</tr>
</tbody>
</table>

The following patients were excluded from analysis of the effect of GVHD and engraftment rate in CGL: 4 who died early, 11 who did not engraft and 2 where engraftment day was not reported. Inclusion of these patients did not affect the remission chances but of course overall survival was then slightly lower. Probability (P) values were calculated by the log-rank method.

There are at least two alternative hypotheses for a possible anti-leukaemic effect of the donor T cells (6). Either they may exert a direct cytotoxic action against the leukaemia cells or there might be an indirect effect mediated by the immune balance between donor and recipient T cells. A low level of host anti-graft activity might delay engraftment and allow a higher proportion of host (including leukaemic) stem cells to repopulate the marrow. On the first
hypothesis we expect relapse to correlate (roughly) with lack of GVHD; on the second, we think that relapse might correlate with slow engraftment.

Detailed analysis of relapse in ALL was impossible because of the small numbers of patients transplanted at any particular phase. For AML transplanted in first remission, Cox linear regression analysis revealed only age as a significant risk factor for relapse (P=0.04), but patients with no GVHD (acute or chronic) also suffered more relapse and this was just significant (P=0.05) after allowing for the higher risk of relapse of older patients. In CGL, there were three risk factors for haematological relapse: higher dose-rate radiation (P=0.02), slow engraftment (P=0.02) and lack of GVHD (P=0.01). The effect of radiation dose rate may not be reliable because of differences in measurement techniques between centres and it could easily have been confounded with other inter-centre variations. The independent correlation of relapse with both lack of GVHD and slow engraftment suggests that both mechanisms of graft-versus-host-leukaemia may be operating in this disease.

Graft rejection is the major problem following T cell depletion in transplants for non-malignant disease and, as we have suggested above, it may also be connected with some of the extra relapse in CGL. We have recently developed the rat IgG2b antibody CAMPATH-1G which has similar specificity to the original IgM, hoping that it would be a useful immunosuppressant for conditioning, by analogy with our successful use of monoclonal antibodies to prevent rejection in a mouse model (7). CAMPATH-1G is active both in complement fixation and antibody dependent cell-mediated killing (8). Three sets of clinical studies suggest that it can eliminate lymphocytes and be immunosuppressive in vivo.

Serotherapy of patients with leukaemia and lymphoma

A total of 16 patients with B cell leukaemias (8) or lymphomas (8) have been treated with CAMPATH-1G (25mg/day for 10 days). The majority had advanced disease which was unresponsive to chemotherapy. In contrast to previous experience with CAMPATH-1H and other antibodies, 13 patients showed tumour regression following antibody treatment. Blood lymphocytes were always cleared, splenomegaly (6 patients) was always improved and bone marrow infiltration (when measured, 7 patients) was always reduced and sometimes (3 patients) cleared of tumour cells. The effect on lymph nodes was not consistent; sometimes they were reduced in size (4 patients) but in others they were unaffected (4 patients) and CNS disease (3 patients) was unaffected. Blood lymphocytes remained low for weeks or months after treatment arguing for cell elimination rather than sequestration.

Treatment of kidney graft rejection

Six patients with acute kidney graft rejection were treated with CAMPATH-1G (5mg/day for 10 days). All had biopsy-proven cellular rejection; in addition four had some vascular changes (presumably antibody-mediated). Three had already received high-dose steroids but without response. In each case the cellular rejection was reversed (judged by biopsy and improved function) though two kidneys were subsequently lost with humoral rejection.

Treatment of graft-versus-host disease

A total of 28 patients with severe GVHD have been treated with CAMPATH-1G
(5mg/day for 10 days). This was a heterogeneous group and hard to analyze in detail. Most had acute GVHD which had not responded to conventional treatment. All showed a response to antibody treatment, judged by improvements in some or all clinical symptoms and biopsy. Some made a good response and became long-term survivors, others suffered recurrence of GVHD after stopping antibody therapy and several died of infectious complications.

Studies are now underway in several centers to assess whether CAMPATH-1G might be useful for prevention of marrow graft rejection. So far, most have concentrated on situations more difficult than transplants from matched siblings, e.g., unrelated donors, partly matched family members or retransplants after graft failure. Although we find the preliminary results encouraging, it is still too early to draw firm conclusions.

Acknowledgements

We thank the Medical Research Council UK for financial support and acknowledge the work of Peter Friend (Department of Surgery) and Martin Dyer (Department of Haematology) in the trials of CAMPATH-1G for treatment of kidney rejection and serotherapy of tumours. We are very grateful to many colleagues and their transplant teams at the following centers for sharing results of marrow transplants and treatment of GVHD. (The numbers of transplants evaluated from each center is shown in brackets.) Most of the data and ideas in this paper were discussed at a meeting of CAMPATH users in Cambridge in Jan 1988. Queen Elizabeth Medical Centre, Birmingham, UK (11); Institute of Haematology Seragno, Bologna, Italy (16); Jules Bordet Hospital, Brussels, Belgium (6); Groote Schur Hospital, Cape Town, South Africa (6); Hopital Cantonal, Geneva, Switzerland (19); Hadassah University Hospital, Jerusalem, Israel (67); Royal Infirmary, Leicester, UK (4); Hammersmith Hospital, London, UK (68); Institute of Child Health, London, UK (7); The London Clinic, London, UK (26); Royal Marsden Hospital, London, UK (11); University College Hospital, London, UK (5); Westminster Hospital, London, UK (35); St. James University Hospital, Leeds, UK (16); Ludwig-Maximilians University Hospital, Munich, FRG (9); Royal Perth Hospital, Perth, Australia (6); King Faisal Hospital, Riyadh, Saudi Arabia (5); University delgi Studi la Sapienza, Rome, Italy (33); Medico Marques de Valdecilla, Santander, Spain (31); University of Ulm, Ulm, FRG (60)

PURGING IN AUTO- AND ALLOGRAFTS: MONOCLONAL ANTIBODIES WHICH USE HUMAN COMPLEMENT AND OTHER NATURAL EFFECTOR MECHANISMS.

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See Table 1 for a list of participating centres.

SUMMARY
CAMPATH-1H is a rat IgM monoclonal antibody which binds to an antigen on all human lymphocytes and monocytes, but which is not present on marrow stem cells [1]. Lymphocytes can be efficiently killed in bone marrow buffy coat preparations using the antibody and donor human serum as a means to avoid graft versus host disease (GVHD) [2]. An analysis of 520 matched sibling bone marrow transplants (BMT) for leukemia demonstrates that T-cell depletion using CAMPATH-1H markedly reduces the incidence and severity of GVHD, but there is an increased risk of graft rejection. In the case of CGL in chronic phase there is also an associated extra risk of relapse, particularly in patients where engrafment may have been compromised [3]. A rat IgG2b antibody of the same specificity as CAMPATH-1H (CAMPATH-1G) was developed which is able to both fix human complement and opsonise lymphocytes in vivo [4]. Initial studies for the prophylaxis of bone marrow rejection in 55 mismatched and matched unrelated donor (MUD) BMHS suggest that CAMPATH-1G treatment of the recipient may reduce, but not eliminate, marrow graft rejection. The broad CAMPATH-1 specificity means that it is also ideal for purging a range of lymphoid malignancies prior to autologous BMT, or even for direct serotherapy of leukemic patients. However, there may be limitations of monoclonal antibody purging using complement or other natural effector mechanisms either in vivo or in vitro; in particular antigenic modulation and an anti-globulin response. Phase 1 studies of 'in vivo purging' with either recombinant antibody or in vitro purging were mostly unsuccessful, most interest has been focussed on ways of trying to improve antibodies by, for example, coupling them to cytotoxic agents. However, our approach has been instead to select monoclonal antibodies which most efficiently use the natural humoral (complement) and cell-mediated (K-cell) effector mechanisms. From a number of studies using rat antibodies against mouse or human lymphocytes, it is now clear that the ability to kill cells both in vitro and in vivo depends on two factors. First, only certain cell surface antigens seem to be effective targets for lysis [7], and second, the monoclonal antibody must be of an appropriate isotype [8]. For example, the CAMPATH-1 antigen (CD52; Fourth International Workshop on Human Leucocyte Differentiation Antigens, Vienna, 1989) is an antigen present on all human lymphocytes and monocytes, and represents a particularly good, non-modulating target antigen for lysis. The rat IgM antibody (CAMPATH-1H) to this antigen is very efficient at lysing cells in vitro with donor human serum as a source of complement, and is therefore our reagent of choice for purging lymphoid cells, including T cells, from bone marrow transplants [1]. However, a series of antibodies to the same antigen (rat IgM, IgG2a, IgG2b, and IgG2c), only the rat IgG2b (CAMPATH-1G) was effective at killing lymphoid cells [4] in vivo or via cell-mediated cytolysis (ADCC). This antibody therefore was the starting point for our attempts to use monoclonal antibodies in vivo for immunosuppression to avoid marrow rejection, and for 'in vivo' purging in lymphoid malignancies.

T-CELL PURGING WITH CAMPATH-1H FOR ALLOGENEIC BONE MARROW TRANSPLANTS

Graft versus host disease
Graft-versus-host disease is the major barrier to allogeneic bone marrow transplantation, but this can be avoided by eliminating T cells from the donor marrow [2]. Since 1983, a large number of centres around the world (for CAMPATH users see Table 1) have contributed to a study of GVHD prophylaxis using CAMPATH-1H and human serum complement for removing T cells [3]. The majority (520) of transplants were from matched sibling donors given to recipients with leukemias as listed in Table 2. Of these, the largest homogeneous group (156) were transplanted for chronic granulocytic leukaemia (CGL) in their first chronic phase (CP1). Donor marrows were depleted of T cells by adding CAMPATH-1H to 0.1mg/ml to buffy coat preparations in a volume of 250ml of balanced salt solution containing C52. Donor human serum was added to 20-25% (v/v) and incubated at 37°C for 30-45 mins. The mean number of residual T cells was then estimated by E-rosette to be 0.7%, with a variation between <0.1% to approximately 1%. As would be predicted, this led to a much reduced incidence and severity of GVHD (Table 3) compared with that expected without T-cell depletion, particularly as half of the patients received no other GVHD prophylaxis. Also encouraging is the very low incidence of chronic GVHD. There was no additional benefit in terms of GVHD for those patients who also received Cyclosporin A, although this group may show a marginally lower incidence of marrow rejection (not significant).

Narrow rejection
Rejection of the T-cell depleted bone marrow remains a major problem for approximately 15% of the transplants, even in this setting of matched sibling donors. It is clear that a solution is needed if unrelated or mismatched BMT are ever to be successful. To this end,
numbers of patients are still small and very heterogeneous in terms of their matching, disease and conditioning (many patients also received TLI), but it does seem that the CAMPATH-1G group as a whole show a much reduced incidence of graft failure (Table 5). However, both in this study, and the few matched sibling CAMPATH-1G patients (Table 3), the rate of rejection is still substantial. One possibility is that the administration of CAMPATH-1G is still not optimal, particularly in relation to all the other conditioning which may compromise some of the effector mechanisms by which the antibody works. Alternatively, it may be that we will need antibodies which are even more potent. With this in mind we have been looking at ways of trying to improve the effectiveness of monoclonal antibodies in vivo.

MONOVALENT ANTIBODIES FOR IN VITRO AND IN VIVO PURGING

Antibody and complement is potentially a very efficient way of killing cells, but there seems to be a number of mechanisms by which cells can protect themselves from such an attack. In particular, bivalent binding to cell surface antigen induces a resistant state and eventual capping and loss of antigen (antigenic modulation). For example, this effect is one of the factors that limit the effective period of GvMT administration [12]. It is possible to make and purify monoclonal antibodies which have one of their two arms inactivated by a light chain with an irrelevant variable region in the binding site [13]. We have shown that such an antibody to CD3 (Monovalent CAMPATH-3) has a greatly increased capacity to kill T-cells with complement in vitro compared to its bivalent parent. The antibody is also effective in vivo, as it was able to deplete the blast cells in a patient with T-cell lymphoma, without causing gross antigenic modulation [5]. It was also able to reverse acute, steroid-resistant cellular rejection episodes in kidney transplant patients [4]. However, two of these patients developed antoglobulin responses which once again would have limited further serotherapy, highlighting another important limitation to the use of rat or mouse monoclonal antibodies in man.

IN VIVO PURGING WITH CAMPATH-1H - TOWARDS THE ULTIMATE AUTOGRFT?

Recent advances in molecular biology have meant that it is now possible to redesign the genes encoding monoclonal antibodies. This led to the development of CAMPATH-1H [14], which is essentially a human IgG1 monoclonal antibody containing just the minimal binding site (the hypervariable loops or complementarity determining regions) from the rat CAMPATH-1G. The humanized antibody is equally, if not more, effective at lysing lymphocytes with human complement or by cell-mediated cytolysis (ADCC). Phase I studies in two patients with non-Hodgkin's lymphoma who had high circulating leukemic cells demonstrated that CAMPATH-1H is extremely potent in vivo (Figure 2), with both patients achieving a complete remission in the peripheral blood, bone marrow, spleen and lymph-nodes [6]. During treatment, bone
narrow function improved and in the patient with neutropenia this resolved. After cessation of treatment, normal oligoclonal B cells began to return in the blood. Neither patient made an antiglobulin response, and when one of the patients showed signs of relapse at approximately six months she was treated with CAMPATH-1H again with equally impressive results and remains well at a further six months. This suggests that humanized monoclonal antibodies are not only extremely effective in vivo, but can be used over extended periods with no loss in efficacy.

The ability of CAMPATH-1H to induce remission in such patients suggests a new approach to the autograft in leukaemia and lymphoma. Rather than to remove and then clean up the diseased bone marrow in vitro, it may be more effective to purge the whole patient, including the marrow, in vivo with monoclonal antibodies. As this treatment is non-toxic to both the patient and the marrow, a conventional autograft then allows cyto-reductive therapy of the patient to clean up any residual leukemic cells which may have escaped the antibody treatment. Such a scheme allows leukemic therapy to concentrate on the patient, where it seems that most relapses originate in current autografting protocols.

CONCLUSIONS

Monoclonal antibodies can utilise natural effector mechanisms such as complement and ADCC to kill cells both in vitro and in vivo, with the one great advantage of simplicity in use. This is effective in controlling both of the major barriers to allogeneic marrow transplantation: graft versus host disease and marrow rejection. In the future the development of even more potent, humanized monoclonal antibodies may lead to new approaches in the treatment of leukaemia and lymphoma, for example, by in vivo purging followed by autografting.

ACKNOWLEDGEMENTS

We are grateful to all the transplant teams at the centres listed in Table 1 for their participation in these studies. We also wish to thank Gilly Martin for preparing the clinical grade monoclonal antibodies. This work was supported by grants from the Medical Research Council and Wellcome Biotech Ltd. CAMPATH is a registered trade mark of Wellcome Foundation.

REFERENCES


FIGURE LEGENDS

Figure 1
CGL-CPI patients with a slow rate of engraftment have an increased rate of relapse.

156 CGL patients in first chronic phase received CAMPATH-1H purged marrow from matched sibling donors. They were divided into two groups: those who grafted (>500 neutrophils/mm³) in less than 25 days (normal engraftment) and those who took 25 or more days to engraft (slow engraftment). The curves show that the probability of remaining in haematologic remission for the two groups at two years is 82% (normal group) vs 58% (slow group); P = 0.03 (Log-rank method).

Figure 2
Induction of remission with CAMPATH-1H in non-Hodgkin lymphoma

A patient with non-Hodgkin lymphoma in leukaemic phase, who was unresponsive to chemotherapy, was given doses of CAMPATH-1H from 1 mg to 20 mg per day (total 126 mg over 30 days). The lymphoma cells (closed triangles) were eliminated from the peripheral blood, while the neutrophils (open triangles), which had been absent at the start of treatment, returned to normal levels. The lymphocytes which appeared in the blood from day 42 were of normal morphological appearance, included both CD3 and CD19 positive cells.

This figure is reproduced with permission from The Lancet (Ref 6).

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<td>St. James University Hospital, Leeds, UK</td>
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<tr>
<td>Ludwig-Maximilians University Hospital, Munich, FRG</td>
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<td></td>
<td></td>
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<tr>
<td>University del Studi di Sapienza, Rome, Italy</td>
<td>36</td>
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<td></td>
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<td></td>
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<tr>
<td>Jules Bordet Hospital, Brussels, Belgium</td>
<td>6</td>
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<td></td>
</tr>
<tr>
<td>Groote Schuur Hospital, Cape Town, South Africa</td>
<td>7</td>
<td></td>
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<tr>
<td>Royal Infirmary, Leicester, UK</td>
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</tr>
<tr>
<td>South Western Regional Transfusion Centre, Bristol, UK</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>University of Wales Medical School, Cardiff, UK</td>
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<td>Royal Devon and Exeter Hospital, Exeter, UK</td>
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<tr>
<td>Royal Free Hospital, London, UK</td>
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<td></td>
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<td>New Addenbrooke's Hospital, Cambridge, UK</td>
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<td>East Hospital, University of Gothenburg, Sweden</td>
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### TABLE 3

**GVHD and graft failure**

<table>
<thead>
<tr>
<th>TOTAL NUMBER BMTs (matched leukaemia)</th>
<th>520</th>
</tr>
</thead>
<tbody>
<tr>
<td>+GVHD prophylaxis</td>
<td>260 (50%)</td>
</tr>
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</table>

**ACUTE GVHD**

<table>
<thead>
<tr>
<th>Grade</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>grade 0</td>
<td>250</td>
</tr>
<tr>
<td>grade 1</td>
<td>69 (18%)</td>
</tr>
<tr>
<td>grade 2</td>
<td>36 (9%)</td>
</tr>
<tr>
<td>grade 3/4</td>
<td>39 (10%)</td>
</tr>
</tbody>
</table>

**CHRONIC GVHD**

- nil: 268
- mild/moderate: 49 (15%)
- severe: 7 (2%)

**GRAFT FAILURE**

- complete: 74 (14%)
- partial: 7 (1%)
- -TLI (379 patients): 66 (17%)
- +TLI (141 patients): 15 (11%)
- -CP1G (501 patients): 79 (16%)
- +CP1G (19 patients): 2 (11%)

Patients are scored 0 for GVHD only if they survived at least 100 days (120 days for chronic GVHD) without GVHD or graft failure.

### TABLE 2

**Breakdown of patients by disease**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL-CR</td>
<td>101</td>
</tr>
<tr>
<td>ALL-REL</td>
<td>16</td>
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<tr>
<td>ALL (unclass)</td>
<td>7</td>
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<tr>
<td>AML-CR</td>
<td>128</td>
</tr>
<tr>
<td>AML-REL</td>
<td>19</td>
</tr>
<tr>
<td>AML (unclass)</td>
<td>7</td>
</tr>
<tr>
<td>CGL-CF1</td>
<td>156</td>
</tr>
<tr>
<td>CGL-other</td>
<td>46</td>
</tr>
<tr>
<td>OTHERS</td>
<td>40</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>520</td>
</tr>
</tbody>
</table>

CR= in complete remission
REL= in relapse
CF1= in first chronic phase

### TABLE 4

**Survival and remission rates for matched BMT in leukaemia**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of patients</th>
<th>Probability of remaining in remission at 2 yr</th>
<th>Actuarial survival at 2 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL-CR1</td>
<td>45</td>
<td>77 ± 8</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>AML-CR1</td>
<td>108</td>
<td>79 ± 7</td>
<td>49 ± 7</td>
</tr>
<tr>
<td>CGL-CF1</td>
<td>156</td>
<td>75 ± 5</td>
<td>64 ± 4</td>
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</tbody>
</table>

CR1 = first complete remission
CF1 = first chronic phase
### Table 5

Graft failure in mismatched and matched unrelated transplants (leukaemia, inborn errors, aplastic anaemia)

<table>
<thead>
<tr>
<th>Match</th>
<th>evaluable</th>
<th>graft</th>
<th>fail</th>
<th>X</th>
<th>P</th>
<th>(chi-squared)</th>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>H</td>
<td>-CPIG</td>
<td>36</td>
<td>19</td>
<td>53</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+CPIG</td>
<td>7</td>
<td>2</td>
<td>29</td>
<td></td>
<td></td>
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<tr>
<td>M</td>
<td>-CPIG</td>
<td>31</td>
<td>21</td>
<td>68</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+CPIG</td>
<td>11</td>
<td>3</td>
<td>27</td>
<td></td>
<td></td>
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<tr>
<td>F</td>
<td>-CPIG</td>
<td>7</td>
<td>3</td>
<td>43</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+CPIG</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>-CPIG</td>
<td>16</td>
<td>6</td>
<td>38</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+CPIG</td>
<td>30</td>
<td>5</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>-CPIG</td>
<td>94</td>
<td>49</td>
<td>54</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+CPIG</td>
<td>55</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

H = related 3 antigen mismatch  M = related, 1-2 antigen mismatch
F = related, phenotypic match  P = unrelated, phenotypic match
+CPIG = patients receiving CAMPATH-1H before grafting with CAMPATH-1M purged marrow.
+CPIG = historical controls receiving CAMPATH-1M purged marrow.
Keystone Symposium on Bone Marrow Transplantation: 19-26 Jan 1992

Proposed abstract for discussion at workshop on "Prevention and treatment of GVHD"

CAMPATH-1 ANTIBODIES FOR PREVENTION OF GVHD AND GRAFT FAILURE

Geoffrey Hale and Herman Waldmann for CAMPATH users, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, UK.

The advantages and drawbacks of T cell depletion are now well known. The use of CAMPATH-1M (IgM) with human complement for in vitro depletion has been one of the simplest and most effective methods, resulting in a low incidence of GVHD, but an increased risk of graft failure. An increased risk of relapse for patients with CGL is also well documented, but 5 year follow-up on patients with acute leukaemia fails to show extra relapse. It has been shown that graft failure is due to radioreistant host T cells so, in an effort to reduce it, we have introduced the IgG antibody CAMPATH-1G into conditioning regimes. This antibody is very effective at depleting lymphocytes in vivo and recent clinical studies show that this results in less graft failure in the context of T cell depletion. CAMPATH-1G alone can also reduce GVHD, either when added to the marrow infusion, or when administered to the patient at the time of transplantation. These findings lead to a new proposal for a simple protocol to avoid the immunological problems of marrow transplantation which we believe will be applicable to transplants from matched unrelated or mismatched donors as well as siblings.

MASTER COPY
DO NOT REMOVE FROM FILE
Professor Peter Jacobs

Elimination of graft-versus-host disease by in-vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1).


A new monoclonal rat anti-human lymphocyte antibody (CAMPATH-1) which lyses cells with autologous human complement was used for depletion of T lymphocytes from human bone-marrow allografts in vitro before transplantation in 11 high-risk patients. HLA-matched siblings were used as marrow donors. T-cell depletion was substantial when measured by E-rosette formation (0-0.18% residual T cells) and immunofluorescence with a monoclonal anti-T-cell antibody (0-0.5%). No anti-graft-versus-host disease prophylaxis was given after transplantation. Rapid engraftment was reported in all patients, and the post-transplantation course was uneventful. No signs of graft-versus-host disease developed in any of the patients, who were observed for a maximum period of 12 months. The method might be suitable for larger-scale studies in high-risk patients. The late graft failure seen in 2 patients may reflect residual host resistance uncompromised by GVHD.

PMID: 6147548 [PubMed - indexed for MEDLINE]
Availability of Campath-1 antibodies for bone marrow transplantation

Recently you have published two papers from the Campath users group showing that Campath-1 antibodies can be successfully used to control graft-versus-host disease and graft rejection in particular situations. Since then we have received many requests from different centres wanting to know whether these antibodies could be made more widely available, so we are writing to explain the present situation.

The original rat CD52 antibodies Campath-1M and Campath-1G have been produced by us in the Therapeutic Antibody Centre for a number of physicians who requested them for clinical trials. Although we aim to provide material of a suitable quality for clinical use, our premises do not have a manufacturers licence nor do the antibodies have a product licence. Our understanding is that in the UK, at least, it is permissible for physicians to use unlicensed products under certain circumstances provided that they obtain approval from the Medicines Control Agency by means of a DDX (doctor's and dentist's exemption).

Until recently the Wellcome Foundation have been developing the humanised antibody Campath-1H for possible use in the treatment of non-Hodgkin's lymphoma and rheumatoid arthritis, and so this antibody might have become commercially available. However, on 27 September 1994 they announced that the development of Campath-1H had been halted because it seemed to have insufficient commercial potential. The possibility of another company taking on the project has not been ruled out.

Meanwhile, we have recently been awarded funding to rebuild and improve our production facilities and hope to open a new production centre in Oxford in 1995. While this work is going on, our ability to supply Campath-1 antibodies will be limited. Although we will continue to support the trials which have already been started, we regretfully have to decide that it is impossible to accept any new requests. We believe that careful scientific evaluation together with commercial development is the best way of ensuring that these reagents are available to the transplant community in the long run. We hope that those teams reading this letter with whom we have not had the pleasure of working will understand our present situation.

G Hale
H Waldmann

1Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QP, and
2Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

References
CD52 antibodies for prevention of graft-versus-host disease and graft rejection following transplantation of allogeneic peripheral blood stem cells

G Hale¹, P Jacobs², L Wood³, WE Fibbe¹, R Barge⁴, N Novitzky⁴, C du Toit⁴, L Abrahams⁴, V Thomas⁴, D Bunjes⁵, C Duncker⁵, M Wiesmeth⁵, D Selleslag⁶, M Hidajat⁶, M Starobinski⁷, P Bird¹ and H Waldmann¹

¹Sir William Dunn School of Pathology, University of Oxford, Oxford, UK; ²Constantiaberg Medi-Clinic, Cape Town, South Africa; ³Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands; ⁴Department of Hematology, Groote Schuur Hospital and the Leukaemia Unit, University of Cape Town, Cape Town, South Africa; ⁵Bone Marrow Transplant Unit, Ulm University Hospital, Ulm, Germany; ⁶Department of Hematology, A-Z-St Jan, Brugge, Belgium; and ⁷Department of Hematology, Hospital Cantonal, Geneva, Switzerland

Summary:

Graft-versus-host disease (GVHD) is a major cause of mortality and morbidity after allogeneic bone marrow transplantation, but can be avoided by removing T lymphocytes from the donor bone marrow. However, T cell depletion increases the risk of graft rejection. In this study, two strategies are used to overcome rejection: (1) use of high doses of stem cells obtained from peripheral blood (PBSC). (2) admixture with a CD52 monoclonal antibody in order to deplete both donor and residual recipient lymphocytes. Two antibodies are compared: CAMPATH-1G (rat IgG2b) and its humanized equivalent CAMPATH-1H (human IgG1). A total of 187 consecutive patients at six centers received PBSC transplants from HLA-matched siblings between 1997 and 1999. A wide spectrum of diseases, both malignant and non-malignant, was included. The recovery of CD3⁺ cells after antibody treatment was close to 100%. The risk of acute GVHD (grade 2 to 4) was 11% in the CAMPATH-1G group and 4% in the CAMPATH-1H group (P = NS). The risk of chronic GVHD (any grade) was 11% in the CAMPATH-1G group and 24% in the CAMPATH-1H group (P = 0.03) but the risk of extensive chronic GVHD was only 2%. The overall risk of graft failure/rejection was 2%, not significantly different between the two antibodies. Antibody treatment was equally effective at concentrations between 10 µg/ml and 120 µg/ml and it made no significant difference to the outcome whether the patients received post-transplant immunosuppression or not (87% did not). Transplant-related mortality in this heterogeneous group of patients (including high-risk and advanced disease) was 22% at 12 months. It is proposed that treatment of peripheral blood stem cells with CAMPATH-1H is a simple and effective method for depleting T cells which may be applicable to both autologous and allogeneic transplants from related or unrelated donors. Special advantages of this approach are the simultaneous depletion of donor B cells (which reduces the risk of EBV-associated lymphoproliferative disease) and the concomitant infusion of CAMPATH-1H to deplete residual recipient T cells and thus prevent graft rejection. Bone Marrow Transplantation (2000) 26, 69-76.

Keywords: CAMPATH-1H; peripheral blood progenitor cells; GVHD; graft failure; HLA-identical sibling; T cell depletion

Transplantation of allogeneic stem cells is a curative therapy for patients with congenital or acquired deficits of hemopoiesis. Combined with high-dose chemotherapy and radiotherapy, it is also a very effective treatment for hematologic malignancies. In recent years, it has become possible to harvest large numbers of stem cells from peripheral blood following mobilization with growth factors, and this is likely to eliminate the need for aspiration of donor bone marrow. However, all forms of allogeneic stem cell transplantation suffer from adverse effects, the most serious being graft-versus-host disease (GVHD), caused by attack of the donor T cells on recipient tissue. Despite the best immunosuppressive therapy with combinations of cyclosporin and methotrexate, this remains a significant cause of morbidity and mortality. If it were not for side-effects such as GVHD, allogeneic stem cell transplantation might be considered for treatment of other conditions, such as autoimmune diseases and induction of tolerance to organ transplants.

GVHD can be prevented by depleting T lymphocytes from the donor stem cells and there are several methods to accomplish this. In 1983 we developed the monoclonal antibody CAMPATH-1M, a rat IgM antibody which recognizes the CD52 antigen. CD52 is abundantly expressed on all human lymphocytes and is an exceptionally good target for cell lysis by antibody with human complement. This provided a simple method for purging the donor T cells which was very effective and gave a significant reduction in GVHD. However, the benefit was offset by an
increased risk of graft rejection by residual host T cells. Animal models showed that this might be overcome by using monoclonal antibodies to deplete residual host T cells. A rat IgG2b CD52 antibody, CAMPATH-1G, effectively depletes human lymphocytes in vivo. Like CAMPATH-1M, it can activate human complement, although this is not sufficient for systemic T cell depletion. Rat IgG2b also binds human Fe receptors and engages cellular killing mechanisms (ADCC). The combination of CAMPATH-1M to T cell deplete donor bone marrow and the intraoperative injection of CAMPATH-1G to ablate residual host immunity overcomes both the problems of GVHD and graft rejection and results in a significantly decreased transplant-related mortality compared with conventional long-term immunosuppressive therapy.

However, a simpler strategy was to use a single dose of CAMPATH-1G added to donor bone marrow to accomplish both objectives at once. Some antibody binds to donor T cells, preventing them for subsequent clearance. The excess antibody is infused along with the bone marrow and gives sufficient depletion of residual host T cells to prevent rejection. We therefore wondered if the same simple method could be used with peripheral blood stem cells. In this case, the number of contaminating T cells is potentially larger and may be quite variable, according to the exact mobilization and collection protocols.

In 1988, a humanized version of CAMPATH-1G was described, namely CAMPATH-1H. This antibody is being developed for the treatment of CLL. CAMPATH-1H is a human IgG1, chosen for its optimal effector function and in principle it should be equally as effective as CAMPATH-1G for elimination of T cells in vivo. There is already a lot of data to support this concept from clinical trials with CAMPATH-1H in CLL, rheumatoid arthritis, multiple sclerosis and organ transplantation. Here, CAMPATH-1G and CAMPATH-1H are compared for use in allogeneic stem cell transplantation and by consideration of the heterogeneity of actual stem cell donations, the robustness of the treatment protocol is assessed.

Collection of peripheral blood stem cells
Donors were treated with G-CSF s.c. at between 5 and 20 μg/kg/24 h in order to achieve a white cell count between 20 and 50 × 10^9 per litre by day 5. Apheresis was started around 2 h after the last dose of G-CSF and continued for up to 6 h. The anticoagulant was ACD-A, typically one volume for every 10 volumes of blood. Based on the ideal body weight of the patient, the target cell dose was either 5 × 10^6 mononuclear cells/kg or 4–5 × 10^6 CD34+ cells/kg. This typically required the processing of 10 to 20 litres of blood. In the event that the target cell dose was not achieved, the apheresis procedure was repeated the following day subject always to the safety and tolerability of the procedure for the donor. No donors were subjected to more than two aphereses. Special procedures were established for donors <35 kg according to local requirements. The volume of each apheresis unit was measured and the concentration of mononuclear cells determined using a suitable automated differential cell counter.

Treatment with monoclonal antibody
CAMPATH-1G or CAMPATH-1H was added to the stem cell concentrate, gently mixed and kept for 30 min at room temperature (18–25°C). The target dose of antibody was either 10 μg or 20 μg according to local protocol, but additional antibody was sometimes added up to a maximum of 60 μg when the volume or number of cells was particularly high. In the event that two apheresis units were collected, standard procedure was to apportion the antibody dose between them. The entire cell suspension was then infused to the recipient over 30 to 60 min without further manipulation according to standard local procedures. Various premedications were used to ameliorate the expected flu-like symptoms due to cytokine release caused by the antibody, including paracetamol (acetaminophen) with either 100 mg hydrocortisone plus 12.5 mg phenergan (promethazine hydrochloride), or prednisone (2 mg/kg) given 1 h before the graft.

Measurement of cell subpopulations
Mononuclear cells were measured with electronic cell counters and calculated as the combination of lymphocytes plus monocytes from differential cell counts. CD34+ progenitors and CD3+ T cells were enumerated by flow cytometry both before and after the antibody treatment. CD34+ cells were measured according to the guidelines of the International Society for Hemotherapy and Graft Engineering using phycoerythrin-labelled CD34 antibody and FITC-labelled CD45 antibody.

Patients
Six transplant centers participated in the study: A-Z St Jan, Bruges; Groote Schuur Hospital, Cape Town; Constantinberg Medi-Clinic, Cape Town; Hopital Cantonal, Geneva; University Hospital, Leiden; Ulm University Hospital, Ulm. They are arbitrarily coded 1–6. Each center recruited consecutive patients provided that they gave informed con-
sent. Data were collected on all patients who received stem cell transplants treated in vitro with CAMPATH-1G or CAMPATH-HI up till May 1999 (a total of 217 patients).

For this analysis, 30 patients were excluded who received transplants from donors other than HLA-matched siblings or who also received CAMPATH-1 antibodies in vivo as part of the conditioning regimen. All categories of disease, conditioning regimens and post-transplant immunosuppression were included, representing the range of normal practice at each center.

**Statistical analysis**

Characteristics of the treatment groups and outcomes were compared using the chi-square test for categorical variables and the Mann Whitney U/Wilcoxon rank sum test for continuous variables. Time-dependent outcomes (engraftment, survival) were compared by the log-rank test. Multifactorial non-linear regression was used to analyze the possible relationship between GVHD and the following covariates: patient sex, patient age, donor sex, type of antibody, post-transplant immunosuppression, antibody dose, antibody concentration, cell dose. These tests were carried out using the ‘FIRST’ suite of statistical software (Serious Statistical Software, Lynwood, South Wirral, UK) or ‘LOGRANK’ software (SP Coblod, University of Oxford, Oxford, UK) running under the RISC operating system on an Acorn Archimedes computer.

**Results**

**Study centers, conditioning regimens and patient characteristics**

The purpose of this study was to test whether a simple single procedure for treatment of donor stem cells could be used with all types of patients. Therefore, consecutive patients were enrolled at each center with no selection according to type or stage of disease, and all other aspects of the transplant procedure (conditioning regimen, prophylactic antibiotics etc.) continued according to the usual local procedures adopted as clinically required according to the status of the patient. Most patients received standard conditioning with cyclophosphamide plus total body irradiation (TBI), but where TBI was not indicated, a combination of chemotherapeutic agents was used (Table 1). Two centers gave supplementary total lymphoid irradiation as anti-rejection prophylaxis. One center used only CAMPATH-1G for treatment of the stem cells, two used only CAMPATH-HI and three switched from the rat to the humanized antibody during the course of the study. Post-transplant immunosuppression with cyclosporin A (CyA) or a combination of CyA and methotrexate (MTX) was used routinely at only two centers. Three patients received donor leukocyte infusions post transplant for prevention or treatment of relapse. Other characteristics of the patient population are summarized in Table 2.

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<th>Centre number</th>
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<th>3</th>
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<tr>
<td>Numbers of patients</td>
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<td>33</td>
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<td>7</td>
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<td>31</td>
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</tbody>
</table>

*Non-TBI regimens consisted of busulfan/cyclophosphamide, busulfan/methotrexate, cyclophosphamide/methotrexate, methotrexate/thiotepa or busulfan/methotrexate/thiotepa.

Protocols normally included standard-dose cyclophosphamide, sometimes with the addition of other anti-leukemia chemotherapies depending on the clinical indication.

Ten patients received additional conditioning with anti-lymphocyte globulin and five received radioimmunotherapy with a radiolabelled CD56 antibody.

*Refers only to T cells given at the time of transplant. Some centers gave donor leukocytes for treatment of relapse.

*Total lymphoid irradiation was given routinely by two centers in addition to TBI. This was increased to 1500 rad for three patients with aplastic anaemia who did not receive TBI.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>80</th>
<th>107</th>
<th>187</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median year of transplant</td>
<td>97 (96-99)</td>
<td>98 (97-99)</td>
<td>98 (96-99)</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>36</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>Male patient</td>
<td>36%</td>
<td>54%</td>
<td>44%</td>
</tr>
<tr>
<td>Male donor</td>
<td>47%</td>
<td>51%</td>
<td>50%</td>
</tr>
<tr>
<td>Median age at transplant</td>
<td>36.5 (6-59)</td>
<td>39 (2-63)</td>
<td>39 (0-61)</td>
</tr>
<tr>
<td>TBI regimen</td>
<td>90%</td>
<td>94%</td>
<td>93%</td>
</tr>
<tr>
<td>TLI regimen</td>
<td>90%</td>
<td>94%</td>
<td>93%</td>
</tr>
<tr>
<td>Post-transplant CyA</td>
<td>22%</td>
<td>7%</td>
<td>13%</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early leukaemia</td>
<td>52%</td>
<td>54%</td>
<td>53%</td>
</tr>
<tr>
<td>Other malignant</td>
<td>36%</td>
<td>37%</td>
<td>37%</td>
</tr>
<tr>
<td>Non-malignant</td>
<td>11%</td>
<td>8%</td>
<td>10%</td>
</tr>
</tbody>
</table>

The extreme range of the data is shown in brackets. Probabilities (P) are calculated by the chi-squared test or Mann Whitney-U/Wilcoxon rank sum test as appropriate. Diseases are classified as: early leukaemia, ALL-CRI, AML-CRI, CML-CP1; other malignant, more advanced leukaemia, lymphoma and myeloma; non-malignant, Fanconi's anaemia, aplastic anaemia and inborn errors.

**Recovery of cells from apheresis**

The yields anticipated a priori for an adult apheresis unit were: volume 200 to 500 ml, total number of mononuclear cells 100 to 300 x 10^6, CD34+ cells 100 to 300 x 10^6. The median actual yields were approximately double the original targets but there were no major differences between the two groups of patients (Table 3). There was a range of
Table 3  Cell recovery after apheresis and treatment with CAMPATH-1 antibodies

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>CAMPATH-1G (1) median (range)</th>
<th>CAMPATH-1H (2) median (range)</th>
<th>Total P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apheresis volume (ml)</td>
<td>384 (84-1130)</td>
<td>384 (NS)</td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells (x10⁶)</td>
<td>571 (113-1853)</td>
<td>567 (0.04)</td>
<td></td>
</tr>
<tr>
<td>CD34⁺ cells (x10⁶)</td>
<td>476 (79-2832)</td>
<td>377 (33-1840)</td>
<td></td>
</tr>
<tr>
<td>CD34⁺ cells (%)</td>
<td>0.5 (0.1-3.0)</td>
<td>0.6 (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td>CD3 cells (x10⁶)</td>
<td>331 (4-2142)</td>
<td>294 (&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>CD3⁺ cells (%)</td>
<td>69 (25-89)</td>
<td>56 (&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Antibody treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody dose (mg)</td>
<td>20 (10-60)</td>
<td>20 (&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>Concentration (µg/ml)</td>
<td>57 (10-240)</td>
<td>56 (0.02)</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>30</td>
<td>30 (NS)</td>
<td></td>
</tr>
<tr>
<td>Temp (°C)</td>
<td>20 (20-25)</td>
<td>20 (NS)</td>
<td></td>
</tr>
<tr>
<td>Cells infused</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells (%)</td>
<td>64 (41-87)</td>
<td>72 (18-164)</td>
<td>0.004</td>
</tr>
<tr>
<td>CD34⁺ cells (%)</td>
<td>131 (32-457)</td>
<td>92 (10-684)</td>
<td>0.01</td>
</tr>
<tr>
<td>CD3⁺ cells (%)</td>
<td>34 (1-87)</td>
<td>27 (5-65)</td>
<td>NS</td>
</tr>
<tr>
<td>Cells infused</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mononuclear cells (x10⁶/kg)</td>
<td>6.7 (3-35)</td>
<td>6.7 (NS)</td>
<td></td>
</tr>
<tr>
<td>CD34⁺ cells (x10⁶/kg)</td>
<td>7.7 (0.3-88)</td>
<td>5.7 (0.2-34)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Probabilities (P) were calculated by the Mann-Whitney-Wilcoxon rank sum test. Differences in the numbers of CD34⁺ cells remained significant even after excluding data from one center which reported lower CD34⁺ counts than others.

Cell recovery following antibody treatment

There were small differences between the two groups, but in both cases the recovery of CD34⁺ cells was very good (Table 3). The median recovery of mononuclear cells was 70% (95% CI 44-99%) and the total number infused was 6.7 x 10⁶ per kg (95% CI 3.5-14 x 10⁶ per kg), which was a little higher than the target of 5 x 10⁶ per kg. The median recovery of CD34⁺ cells was 99% (95% CI 35-666%) and the total number infused was 6.3 x 10⁶ per kg (95% CI 19 x 10⁶ per kg), i.e. close to 1% of the mononuclear cells. The median recovery of CD3³ cells was 26% (90% CI 5-65%). It was not expected that all the T cells would be lysed in vivo.

Engraftment and graft failure

Univariate analyses of outcome are shown in Table 4. Recovery of neutrophils was rapid, as expected for peripheral blood stem cell transplants. The median day to reach 500 neutrophils/µl was day 13 (range 10-44) for the CAMPATH-1G group and day 12 (range 8-30) for the CAMPATH-1H group. There was one case of early graft failure (never reached 500 neutrophils/µl) and one case of late graft failure in each group.

Graft-versus-host disease

The incidence of both acute and chronic GvHD was higher in the CAMPATH-1H group compared with the CAMPATH-1G group, almost entirely as a result of a higher frequency of grade 1 acute GvHD or mild/moderate chronic GvHD (Table 4). However, neither the antibody dose nor the final antibody concentration had a significant effect on the incidence of GvHD. This is exemplified in...
Table 4  Outcome according to treatment group

<table>
<thead>
<tr>
<th>% Probability (95% confidence interval)</th>
<th>P&lt;sub&gt;12&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CAMPATH-1G (I)</strong></td>
<td><strong>CAMPATH-1H (II)</strong></td>
</tr>
<tr>
<td>0.5 x 10^6 neutrophils</td>
<td></td>
</tr>
<tr>
<td>by day 13</td>
<td>52 (37.68)</td>
</tr>
<tr>
<td>by day 21</td>
<td>90 (81.99)</td>
</tr>
<tr>
<td>by day 30</td>
<td>92 (64.100)</td>
</tr>
<tr>
<td>by day 60</td>
<td>97 (93.100)</td>
</tr>
<tr>
<td>Graft failure</td>
<td></td>
</tr>
<tr>
<td>at 1 month</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>at 12 months</td>
<td>3 (0.6)</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td></td>
</tr>
<tr>
<td>grade I</td>
<td>23</td>
</tr>
<tr>
<td>grade II</td>
<td>7</td>
</tr>
<tr>
<td>grade III-IV</td>
<td>4</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td></td>
</tr>
<tr>
<td>mild-moderate</td>
<td>11</td>
</tr>
<tr>
<td>extensive</td>
<td>0</td>
</tr>
<tr>
<td>Transplant-related mortality</td>
<td></td>
</tr>
<tr>
<td>at 12 months</td>
<td>21 (10.32)</td>
</tr>
<tr>
<td>at 24 months</td>
<td>27 (13.42)</td>
</tr>
</tbody>
</table>

The outcome is reported, together with the 95% confidence interval. Probabilities were calculated by the chi-squared test (GVHD) or the univariate log-rank test (other outcomes). They do not take into account any potential covariates.

Figure 1, where the mean GVHD grade is plotted against antibody concentration. Similar results (not shown) were obtained when mean grades for acute or chronic GVHD were plotted against total antibody dose or total cell dose. Furthermore, the use of post-transplant immunosuppression had no significant impact on the frequency of GVHD. The influences of potential covariates was examined by multivariate linear regression with acute GVHD as the dependent variable and the following independent variables: patient sex, patient age, donor sex, type of antibody, post-transplant immunosuppression, antibody dose, antibody concentration, cell dose. The only factor found to be correlated with acute GVHD was the patients age – not surprisingly, there was an increasing risk of GVHD in older patients.

Mortality

Transplant-related mortality is defined as death from any cause other than relapse of the original disease. There was no significant difference between the groups (Table 4). Because of the relatively short follow-up and the disparate diseases treated, there was no attempt to analyze relapse, overall survival or leukemia-free survival. The causes of death are listed in Table 5. There was one death from EBV-associated lymphoproliferative disease. There were more deaths from infection in the CAMPATH-1H group (13%) than the CAMPATH-1G group (6%), although the difference is not statistically significant due to the relatively small numbers. As expected, there were relatively fewer deaths among the patients transplanted for early leukemia, but with the small numbers and short follow-up of this study, the differences were not statistically significant.

Comparison with reference groups of patients

No large reports are available from the transplant registries or other sources with which to compare these data. We selected three comparison groups of transplants from HLA-identical siblings in order to put the results into a clinical context. (1) Bone marrow transplants treated with CAMPATH-1G ex vivo according to the present protocol. The patients are described in Refs 3, 4, 11 but the most recently updated data (previously unpublished) have been used. (2)
PBSC transplants where GVHD prophylaxis consisted of either cyclosporin or tacrolimus combined with either methotrexate or prednisolone. Data from eight separate single-center studies were combined as described by Besinger and Buckner. The follow-up was short and so there are uncertainties in the calculation of chronic GVHD and transplant-related mortality. (3) PBSC transplants which had been T cell-depleted by positive selection of CD34+ cells by either an immunoadsorption or an immunomagnetic technique. The comparative data are presented in Table 6.

Discussion

Treatment of bone marrow with CAMPATH-1G has been established for some years as an effective method for depletion of donor T cells to prevent GVHD, without incurring a high risk of graft rejection. In principle, grafting is expected to be faster and more reliable from peripheral blood stem cells. However, it was not obvious whether the larger numbers of contaminating T cells could be dealt with as effectively by the antibody treatment. Furthermore, we wanted to test whether CAMPATH-1G could be replaced by the humanized version, CAMPATH-3H. This prospective analysis of PBSC transplants at the participating centers was carried out to compare the outcomes according to the type of antibody used. Although certain aspects of the antibody treatment protocol were standardized in advance (eg, time and temperature of treatment), other potentially important parameters were not so tightly controlled because of the varying logistical requirements of different patients. This provided an opportunity to determine the importance of factors such as antibody dose, concentration and post-transplant immunosuppression; in effect, a natural experiment to test the robustness of the procedure.

<table>
<thead>
<tr>
<th>Table 6</th>
<th>Comparison with other series of transplants</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>CAMPATH</td>
</tr>
<tr>
<td></td>
<td>BMT</td>
</tr>
<tr>
<td>Number of patients</td>
<td>187</td>
</tr>
<tr>
<td>Early leukemia</td>
<td>53%</td>
</tr>
<tr>
<td>Early death</td>
<td>6.3</td>
</tr>
<tr>
<td>Graft failure</td>
<td>2%</td>
</tr>
<tr>
<td>Day to 0.5 x 10^9 neutrophils</td>
<td>13</td>
</tr>
<tr>
<td>Acute GVHD (grade 2 &amp; 4)</td>
<td>75%</td>
</tr>
<tr>
<td>Acute GVHD (grade 3 &amp; 4)</td>
<td>2%</td>
</tr>
<tr>
<td>Chronic GVHD (any grade)</td>
<td>19%</td>
</tr>
<tr>
<td>Chronic GVHD (extensive)</td>
<td>2%</td>
</tr>
<tr>
<td>Transplant mortality at 1 year</td>
<td>23%</td>
</tr>
</tbody>
</table>

Outcomes according to alternative transplant protocols are compared with the outcomes in this study, (1) Bone marrow transplants treated with CAMPATH-1G ex vivo. (2) PBSC transplants with drug-based GVHD prophylaxis. Data marked * are incomplete due to short follow-up or limited reporting. (3) PBSC transplant T cell-depleted by positive selection of CD34+ cells.


tant antibody concentration in the apheresis bag varied from 6 to 240 µg/ml (median 56 µg/ml, 90% CI 10–120 µg/ml). The concentration of CAMPATH-1G or CAMPATH-3H required for 90% saturation of the CD52 antigen is approximately 10–20 µg/ml. However, maximal cell-mediated killing (ADCC) can be achieved with as little as 0.1 µg/ml. Therefore, in theory, even the lowest concentrations of antibody should have been adequate to opsonize the donor T cells for lysis in vivo.

Recoveries of mononuclear cells and CD34+ cells were consistently good with this procedure, which is not surprising, considering the simplicity of the manipulations. It is well documented that CAMPATH-1 antibodies spare hematopoietic colony-forming cells. However, there is a subpopulation of CD34+ cells in bone marrow which is also CD52- and CD34+, most probably lymphoid progenitors.

Treatment of bone marrow with CAMPATH-1G or CAMPATH-3H can result in depletion of this subpopulation, but in peripheral blood CD34+ cells a similar CD52-CD34+ subpopulation was not detected. In an experimental study, the concentrations of cells and antibody were systematically varied over a range corresponding to that achieved in clinical practice, but without effect on the depletion of lymphocytes or recovery of CD34+ cells.

It was not expected that treatment with CAMPATH-1G or CAMPATH-3H would result in extensive depletion of T cells prior to infusion into the patient. Although both antibodies can activate human complement, the conditions were not optimized for complement-mediated lysis. Some degree of depletion did occur in vivo, but the extent was highly variable (13–99%), probably because the amount of residual plasma was not specifically controlled. We believe that the main depleting effect occurs in vivo by antibody-dependent cellular effector mechanisms, after the mixture of antibody and cells has been infused.

GVHD was well controlled in the majority of patients, irrespective of the antibody concentration or the use of post-transplant immunosuppression. The overall incidence of acute and chronic GVHD was significantly lower than expected when CAMPATH-1 antibodies were used in previous studies for T cell depletion of bone marrow transplants and is also similar to that seen using other effective methods for depleting donor T cells. However, there was a higher incidence of grade I acute GVHD and of mild/moderate chronic GVHD in patients who received CAMPATH-3H, despite the fact that the average antibody dose was slightly higher. The reason for this is not known, although it is notable that the starting concentration of T cells in the CAMPATH-3H group was significantly higher. Limited GVHD has not been a major clinical problem; in fact, there is some reason to believe that it may provide a desirable graft-versus-leukemia effect.

The incidence of graft failure or graft rejection in this series of patients was low. Engraftment is probably facilitated by the larger number of stem cells available from peripheral blood compared with bone marrow. In addition, the surplus CAMPATH-1 antibody infused with the transplant probably contributes by depleting any residual host lymphocytes which have been spared by the conditioning regimen. In this regard, the present approach is superior to
those methods which rely purely on in vitro means, such as
magnetic beads, for removing donor T cells. As with most clinical studies of stem cell transplantation,
there are never sufficient patients to allow prospective ran-
domized trials for comparing different treatments. Instead,
we are obliged to rely on historical comparisons and a
registry style of statistical analysis to elucidate significant
trends. Only the most obvious conclusions can be drawn
from the type of comparison presented in Table 6, since we
have to acknowledge that the groups of patients are not
necessarily equivalent in terms of prognostic factors. How-
ever, each group involved consecutive patients treated in
at least six different centers, so the data should be representa-
tive of typical clinical experience. The average number of
CD34+ cells infused in this study was similar to that for
unmanipulated PBSB and about twice that for CD34+ selec-
ted PBSB where the recovery is typically about 50%.
Clearly, T cell depletion results in a significant reduction
in the risks of acute and chronic GVHD, and this is most
striking in the CAMPATH groups because little or no post-
transplant immunosuppression was used, whereas all of
the patients in the CD34+ selected group also received post-
transplant CyA. The risk of graft failure is lower for T cell
depleted PBSB transplants compared with T cell-depleted
bone marrow transplants, probably because of the larger
dose of stem cells, which also results in significantly faster
engraftment. However, the use of CAMPATH-1G or CAM-
PATH-H in PBSB transplants has not been associated with
a reduction in transplant-related mortality. Whereas
deaths from complications of GVHD are greatly reduced
by T cell-depletion, it appears that there is still a risk from
infectious complications.

Thus the two outstanding problems are leukemia relapse
and immune reconstitution. An increased risk of relapse is
likely for patients with CML who received T cell-depleted
allogeneic transplants compared with recipients of T cell
replete transplant. In future, this might be overcome by
controlled infusion of donor leukocytes, but this needs to
be done without risking severe GVHD. Any increase in
relapse risk for patients with acute leukemia is modest, and
probably compensated by the reduction in GVHD. In
previous studies we observed that patients who received T
cell-depleted bone marrow transplants were at risk from
infections mainly during the first year post transplant.
After that the 10 year actuarial risk of death from infection
was only 3.5% (CH and CAMPATH users, unpublished work,
1998). Nevertheless, immune reconstitution after T cell-
depleted PBSB transplants is an issue of concern and needs
to be studied in much more detail over a long term. Such
a study has been carried out on 20 T cell-depleted bone
marrow transplants by one of the present centers. Immu

References

5. Bunjes D, Heit W, Arnold R et al. Evidence for the involvement of host-derived OKT3-positive T cells in the rejection of EBV and consequent lymphoproliferative disease (BLPD), possibly leading to fatal lymphoma. There was one such case in this series of 187 patients. In a published study of 2582 bone marrow transplants T cell-depleted with CAMPATH-1 antibodies, the risk of BLPD was only 1.3%, hardly different from results for T cell replete transplants, but in stark contrast to the high incidences reported following
some other forms of T cell depletion. The difference may be due to the fact that CAMPATH-1 antibodies target donor B cells equally as well as T cells thus reducing the virus load or the virus target or both.

Treatment with CAMPATH-1H is a simple and reliable
method for prevention of GVHD and graft failure following
allogeneic peripheral blood stem cell transplantation. The
procedure results in a good yield of CD34+ cells and effec-
tive control of GVHD under a wide range of conditions. It
may be suitable not only for allogeneic transplants but also
for depletion of T cells in autologous transplant procedures
for autoimmune diseases. During the period of this study
there were very few PBSB transplants from volunteer unre-
lated donors. However, CAMPATH-1 antibodies have
proved to be effective in controlling GVHD following unre-
lated donor marrow transplantation so we hope that the
present approach may also be useful if and when donations
of blood stem cells from unrelated donors become more
widely used. The important issue of immune reconstitution
needs to be addressed and the possibility of using donor
lymphocyte infusions should be considered especially if
methods can be developed to manipulate them to
selectively remove alloreactivity.

Acknowledgements

Antibody production for these studies was supported by the UK Medical Research Council, LeukoSite Inc, and EP Abraham's Trust. We are indebted to many colleagues who played an important part in the production of antibodies, care of patients, data collection and analysis, including the staff of the Therapeutic
Antibody Centre, University of Oxford, also Bernard Chapuis,
Claudine Helg, Corinne Charrin, Colette Grand, and Kate Schoonooghe.

5

Bone Marrow Transplantation


24 Williams RJ, Clarke E, Blair A et al. Impact of T-cell depletion and CD34+ cell recovery using humanized CD52 monoclonal antibody (CAMPATH-1H) in bone marrow and PBSC collections: comparison with CAMPATH-1M and CAMPATH-1G. *Cytotherapy* (in press).


Specificity of monoclonal antibody Campath-1.

Hale G, Waldmann H, Dyer M.

PMID: 3048490 [PubMed - indexed for MEDLINE]
In vivo use of Campath-1G to prevent graft-versus-host disease and graft rejection after bone marrow transplantation

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¹Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands and ²Department of Pathology, University of Cambridge, Cambridge, UK

Summary:

Twenty-two patients (16 male, six female; median age 34 years, range 16–49) with acute myeloid leukemia (1st complete remission (CR), n=9), acute lymphocytic leukemia (1st CR, n=5), chronic myeloid leukemia (chronic phase n=5, accelerated phase n=1), malignant lymphoma (n=1) and myeloma (n=1) were transplanted with unmanipulated donor bone marrow after standard conditioning including the monoclonal antibody Campath-1G daily from day -4 to day 0. No further graft-versus-host disease (GVHD) prophylaxis was given. All patients engrafted and neither graft failure nor rejection were observed. Acute GVHD grade I (skin) was seen in 12 out of 21 patients at risk. Acute GVHD grade II (skin) occurred in two patients. Severe GVHD (grade III, IV) of the gut, liver and skin developed in two patients. The overall incidence of severe acute GVHD (II-IV) was 19% of the patients at risk. Chronic GVHD (skin only) was seen in eight patients (42%) (six of extensive severity). A total of 14 patients died, the causes being relapse (four), direct cytotoxic drug toxicity (one), aGVHD (two), disseminated varicella zoster (one), systemic tuberculosis (one), interstitial pneumonitis (three) and veno-occlusive disease (two). These results indicate that the intravenous administration of Campath-1G may have reduced the incidence of severe acute GVHD without the occurrence of graft failure. However, the incidence of chronic GVHD does not appear to have decreased.

In the past decade ex vivo depletion of mature T lymphocytes from a bone marrow graft was been successful in preventing acute graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT).¹ The incidence of acute GVHD (grade II-IV) decreased from at least 80% for patients receiving no prophylactic treatment at all to 10–25% when T cells were depleted from the bone marrow.² A variety of physical and immunological techniques have been used to deplete the marrow of T cells.³–⁵ The most widely used monoclonal antibody is the rat IgM monoclonal antibody Campath-1M which recognizes a heterogeneous 23–30 kDa glycoprotein expressed by all lymphocytes and monocytes.⁶ In the presence of human complement it may effectively deplete T cells from bone marrow grafts in vitro,⁷ resulting in less than 20% acute GVHD among patients at risk.

However, although in vitro T cell depletion prevents aGVHD, it has resulted in an increased incidence of early and late marrow graft failure and leukemic relapses. Depending on the degree of compatibility between donor and recipient the incidence varies from 10% to more than 30%.⁸,⁹ Alternative approaches, including more intensive immunosuppression as a means of conditioning, increasing the number of marrow cells in the graft, and less complete removal of T cells, have been explored. Attempts to apply more intensive conditioning regimens have caused increased toxicity, which counteracted the positive effects on engraftment and relapse rates, whereas leaving more T cells in the graft resulted in more severe GVHD.⁹

In mice, Cobbold et al.¹⁰ showed that engraftment of in vitro T cell-depleted donor bone marrow may be promoted by the systemic administration of anti-T lymphocyte antibodies to the recipient prior to BMT, presumably as a result of in vivo depletion of recipient lymphocytes capable of rejecting the graft. Thierfelder et al.¹¹ showed that a single injection of the rat monoclonal antibody anti-Thy-1 of the IgG2b isotype after irradiation but before marrow infusion prevented GVHD mortality and suppressed host-versus-graft reactivity so that the radiation dose necessary after engraftment of mismatched donor cells could be reduced.

Clinical data on the effective depletion of lymphocytes in vivo by monoclonal antibodies are scarce.¹²–¹⁶ One of the most lytic of the rodent antihuman lymphocyte antibodies so far is the recombinant IgG2b anti pan-lymphocyte antibody (Campath-1G).²,¹¹ It can be given intravenously and produces a profound depletion of lymphocytes from blood, spleen and bone marrow. It has been applied successfully in the treatment of patients with progressive lymphoma and chronic lymphocytic leukemia without major complications.¹⁷

We report the results of a study on the intravenous administration of Campath-1G to patients undergoing
transplantation of an unmodified matched sibling donor bone marrow graft. The aim of this study was to prevent acute GVHD by in vivo elimination of donor T cells from the graft and simultaneously to prevent rejection by elimination of residual antidonor reactive T cells from the recipient.

Patients and methods

Study group

To investigate the effect of Campath-1G on the number of circulating lymphocytes and to study the pharmacokinetics of the antibody, 12 patients who underwent allogenic BMT between September 1987 and February 1988 received intravenous Campath-1G just before the conditioning regimen was started. Subsequently, between February 1988 and April 1990, a further 22 patients who required a bone marrow graft from an HLA-matched sibling donor entered a phase II study to assess whether Campath-1G could be used during the conditioning phase for depletion of immunocompetent recipient lymphocytes and to determine whether any effect on GVHD incidence, graft rejection and infection pattern could be detected. Clinical features such as age, sex and viral status of donor and recipient, diagnosis and stage of the disease and interval between diagnosis and BMT are presented in Tables I and III. The group comprised 16 male and six female patients with a median age of 34 year (range 16–49). The median age of the donors was 30 years (range 14–48), 14 of them were sex-matched with the recipient. The protocol was approved by the Leiden Internal Review Board. All patients entering the study had given informed consent.

Study protocol

The preparatory regimen consisted of intravenous cyclophosphamide (60 mg/kg/body weight per day on days -6 and -5) and single-dose total body irradiation (9 Gy, 25 cGy/min on day -1) for 18 patients. Because of extensive prior radiotherapy four patients followed a conditioning regimen consisting of oral busulfan at a daily dose of 1 mg/kg body weight four times daily on days -9 to -6, and intravenous cyclophosphamide at a daily dose of 50 mg/kg body weight on days -5 and -4. In both cases Campath-1G at a daily dose of 5 mg in 500 ml 5% glucose was given as a 2–6 h infusion on days -4, -3, -2, -1 and 0. Unmodified bone marrow cells (mean cell number 2.1 × 10^10/kg body weight) were infused within 4 h of the last Campath-1G infusion. Intravenous methylprednisolone (250 mg daily) was given to suppress severe side-effects during the administration of cyclophosphamide and Campath-1G. All patients were nursed in high-efficiency particulate air (HEPA)-filtered isolation units, received a diet with a very low bacterial content, underwent prophylactic selective antibiotic decontamination of the digestive tract and had a central venous catheter. Continuous monitoring of the bacterial, fungal and viral status of the recipient was performed. Supportive treatment included broad-spectrum antibiotics, fungostatic or virostatic agents.

Table I  Characteristics of patients and donors

<table>
<thead>
<tr>
<th>UPN</th>
<th>Sex (M/F)</th>
<th>Age (years)</th>
<th>Diagnosis/Stage</th>
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</thead>
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<tr>
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<td>M</td>
<td>43</td>
<td>Ph^+ ALL, 1st CR</td>
</tr>
<tr>
<td>041</td>
<td>M</td>
<td>34</td>
<td>Sec. AML (M2), 1st CR</td>
</tr>
<tr>
<td>231</td>
<td>M</td>
<td>44</td>
<td>CML, chronic phase</td>
</tr>
<tr>
<td>161</td>
<td>F</td>
<td>28</td>
<td>Sec. AML (M2), 1st CR</td>
</tr>
<tr>
<td>151</td>
<td>M</td>
<td>38</td>
<td>CML, acc. phase</td>
</tr>
<tr>
<td>181</td>
<td>M</td>
<td>44</td>
<td>Sec. AML, 1st CR</td>
</tr>
<tr>
<td>201</td>
<td>F</td>
<td>42</td>
<td>AML, 1st CR</td>
</tr>
<tr>
<td>131</td>
<td>M</td>
<td>27</td>
<td>Ph^+ ALL, 1st CR</td>
</tr>
<tr>
<td>171</td>
<td>M</td>
<td>35</td>
<td>AML (M2), 1st CR</td>
</tr>
<tr>
<td>211</td>
<td>M</td>
<td>29</td>
<td>AML (M1), 1st CR</td>
</tr>
<tr>
<td>251</td>
<td>M</td>
<td>38</td>
<td>NHL (LB), 1st CR</td>
</tr>
<tr>
<td>261</td>
<td>F</td>
<td>16</td>
<td>AML (M5), 1st CR</td>
</tr>
<tr>
<td>271</td>
<td>M</td>
<td>27</td>
<td>CML, chronic phase</td>
</tr>
<tr>
<td>291</td>
<td>M</td>
<td>34</td>
<td>T-ALL, 1st CR</td>
</tr>
<tr>
<td>721</td>
<td>M</td>
<td>26</td>
<td>T-ALL, 1st CR</td>
</tr>
<tr>
<td>681</td>
<td>M</td>
<td>23</td>
<td>T-ALL, 1st CR</td>
</tr>
<tr>
<td>711</td>
<td>M</td>
<td>24</td>
<td>CML, chronic phase</td>
</tr>
<tr>
<td>701</td>
<td>M</td>
<td>23</td>
<td>CML, chronic phase</td>
</tr>
<tr>
<td>741</td>
<td>M</td>
<td>30</td>
<td>AML (M2), 1st CR</td>
</tr>
<tr>
<td>781</td>
<td>F</td>
<td>49</td>
<td>CML, chronic phase</td>
</tr>
<tr>
<td>851</td>
<td>F</td>
<td>28</td>
<td>AML (M5), 1st CR</td>
</tr>
<tr>
<td>881</td>
<td>F</td>
<td>37</td>
<td>Myeloma, stage III</td>
</tr>
</tbody>
</table>

M=male; F=female; CR=complete remission; Ph=Philadelphia chromosome; AML=acute myeloid leukemia; ALL=acute lymphoblastic leukemia; CML=chronic myeloid leukemia; NHL=non-Hodgkin’s lymphoma (lymphoblastic type); (LB)

*Interval between diagnosis and bone marrow transplantation in months
for documented infection and fever, leukocyte-free platelet and red blood cell transfusions to maintain the platelet count above 10 × 10⁹/l and the hemoglobin concentration above 5–6 mmol/l. All blood products were screened for cytomegalovirus (CMV) and were irradiated (20 Gy). Patients of CMV-negative recipient-donor combinations received transfusions of CMV-negative blood products. No additional GVHD prophylaxis was given.

Engraftment was measured by peripheral blood counts and marrow examinations. IgG-class antibodies against herpes simplex virus (HSV) and varicella-zoster virus (VZV) and the capsid antigens of Epstein-Barr virus (EBV) were assessed using an indirect immunofluorescence assay. IgM class antibodies against HSV were detected using an indirect immunofluorescence technique. IgG and IgM class antibodies against cytomegalovirus (CMV) late antigen were assessed with an enzyme-linked immunosorbent assay (ELISA). The diagnosis of active HSV or VZV infection was based on clinical symptoms and, in most cases, confirmed by virus isolation. Active CMV infection was diagnosed on the basis of characteristic symptoms and appearance of the immediate early antigen (IEA) in the blood.

Acute GVHD was assessed according to the Seattle grading system. In case of symptoms of acute GVHD, methylprednisolone was started at a total daily dose of 10 mg/kg body weight. Chronic GVHD was treated with cyclosporin, methotrexate, total lymphoid irradiation or PUVA therapy alone or in combination.

Serum levels of Campath-1G were determined using an immunofluorescence assay. Serum samples were incubated with K422 cells (a B cell line which expresses high levels of the Campath-1 antigen) in the presence of 5 mM EDTA (to prevent complement activation). Bound antibody was detected with FITC-anti-(rat Ig). A standard curve was constructed using known dilutions of Campath-1G in normal human serum and the relationship between mean fluorescence intensity and antibody concentration was determined so that the antibody concentration in the test samples could be calculated.

Results

Pharmacokinetics and toxicity of intravenous Campath-1G

In the preliminary study, 12 patients received Campath-1G intravenously as a 4–6 h infusion for 5 days before the start of the conditioning regimen. Complete blood cell counts and the levels of Campath-1G were determined in daily blood samples. All blood lymphocytes had disappeared within 24 h (data not shown). The levels of Campath-1G were derived from measurements in 12 patients. In 10 patients samples were available from prior to Campath-1G infusion. At various time points during Campath-1G infusion or after Campath-1G infusion, samples were collected, i.e. at each time point, 2–4 samples were available, originating from different patients. All concentrations in Figure 1 represent means of multiple measurements derived from different patients. No kinetic studies in single patients have been performed. Levels of Campath-1G increased to a maximum on the fifth day of infusion. Detectable levels were present up to 24 h after discontinuation of the infusion. Except for fever and chills in a few patients on the first day of administration no side effects were observed. This might be due to the concomitant administration of methylprednisolone.

Effect of Campath-1G on engraftment and GVHD

Effect of Campath-1G on incidence of infection

The rate of reactivation of herpes virus infections is shown in Table III. None of the patients exhibited a clinical reactivation of the EBV virus infection. HSV reactivation within the first weeks after BMT was seen in eight patients, two of whom were seronegative before transplantation and received bone marrow from a seropositive donor. The overall incidence was 40% of the patients at risk. All patients were treated successfully with intravenous acyclovir. CMV reactivation, as measured by the appearance of the immediate early
antigen in the blood in addition to clinical symptoms compatible with a CMV infection, was diagnosed in seven of 15 patients (47%) who had a positive serology or were seronegative but received marrow from a seropositive donor. They were treated with ganciclovir (DHPG). Only one of the seven died, due to CMV pneumonia. Four patients experienced a generalized varicella zoster infection, which in spite of treatment with high-dose acyclovir was fatal in one case. One patient was seronegative before BMT but received marrow from a seropositive donor and acquired a VZV infection.

**Table IIa** Transplantation characteristics

<table>
<thead>
<tr>
<th>UPN</th>
<th>Conditioning regimen</th>
<th>No. of BM cells (x 10^6/Kg BW)</th>
<th>Engraftment (days after BMT)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Gradedocytes &gt; 0.5 x 10^9/L</td>
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<tr>
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<td>BU/CY/Camphath-1G</td>
<td>1.7</td>
<td>17</td>
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<tr>
<td>041</td>
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<td>2.0</td>
<td>13</td>
</tr>
<tr>
<td>231</td>
<td>CY/TBI/Camphath-1G</td>
<td>2.0</td>
<td>35</td>
</tr>
<tr>
<td>161</td>
<td>BU/CY/Camphath-1G</td>
<td>2.2</td>
<td>19</td>
</tr>
<tr>
<td>151</td>
<td>CY/TBI/Camphath-1G</td>
<td>2.0</td>
<td>49</td>
</tr>
<tr>
<td>181</td>
<td>BU/CY/Camphath-1G</td>
<td>3.0</td>
<td>18</td>
</tr>
<tr>
<td>201</td>
<td>CY/TBI/Camphath-1G</td>
<td>2.0</td>
<td>29</td>
</tr>
<tr>
<td>131</td>
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<tr>
<td>171</td>
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<td>45</td>
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<tr>
<td>211</td>
<td>CY/TBI/Camphath-1G</td>
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<td>32</td>
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<td>251</td>
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<td>26</td>
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<td>25</td>
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<td>32</td>
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<td>15</td>
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<td>29</td>
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<td>681</td>
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<td>2.0</td>
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<td>781</td>
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<tr>
<td>851</td>
<td>CY/TBI/Camphath-1G</td>
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<td>881</td>
<td>CY/TBI/Camphath-1G</td>
<td>2.0</td>
<td>Too early, Died on day: 20</td>
</tr>
</tbody>
</table>

BU = busulanan; CY = cyclophosphamide; TBI = total body irradiation

**Table IIb** Grade, date of onset and severity of graft-versus-host disease

<table>
<thead>
<tr>
<th>UPN</th>
<th>Camphath concentration (µg/ml at day 0)</th>
<th>Acute GVHD*</th>
<th>Severity</th>
<th>Chronic GVHD</th>
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<td>Grade</td>
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<td>+</td>
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<td>-</td>
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<td>-</td>
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<td>I</td>
<td>18</td>
<td>+</td>
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<td>131</td>
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<td>I</td>
<td>32</td>
<td>++</td>
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<tr>
<td>171</td>
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<td>I</td>
<td>20</td>
<td>+</td>
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<tr>
<td>211</td>
<td>ND</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>251</td>
<td>2.4</td>
<td>II</td>
<td>19</td>
<td>++</td>
</tr>
<tr>
<td>261</td>
<td>0.2</td>
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<td>19</td>
<td>++</td>
</tr>
<tr>
<td>271</td>
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<td>II</td>
<td>30</td>
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</tr>
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<td>291</td>
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<td>22</td>
<td>+</td>
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<tr>
<td>721</td>
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<td>I</td>
<td>25</td>
<td>-</td>
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<tr>
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<td>III</td>
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<td>+</td>
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<tr>
<td>711</td>
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<td>14</td>
<td>+ ++</td>
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<tr>
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<td>-</td>
</tr>
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<td>I</td>
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</tr>
<tr>
<td>851</td>
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<td>I</td>
<td>28</td>
<td>+</td>
</tr>
<tr>
<td>881</td>
<td>ND</td>
<td>(0)</td>
<td>NA</td>
<td>Too early</td>
</tr>
</tbody>
</table>

NA = not applicable; ND = not done
*According to the Scattie grading system

pneumonitis. Four patients experienced a generalized varicella zoster infection, which in spite of treatment with high-dose acyclovir was fatal in one case. One patient was seronegative before BMT but received marrow from a seropositive donor and acquired a VZV infection.
Table III  Reactivation of viral disease in relation to pretransplant serology

<table>
<thead>
<tr>
<th>Reactivation of viral disease*</th>
<th>Pretransplant serology</th>
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<tr>
<td></td>
<td>n</td>
<td>Only recipient positive</td>
<td>Only donor positive only</td>
<td>Recipient donor positive</td>
<td>Recipient donor negative</td>
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<td>HSV Yes</td>
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<td>1</td>
<td>2</td>
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<td>0</td>
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<tr>
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<td>1</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td></td>
</tr>
<tr>
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<td>6</td>
<td>0</td>
<td>1</td>
<td>0</td>
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</tr>
<tr>
<td>CMV No</td>
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<td>1</td>
<td>3</td>
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<td>0</td>
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<td>VZV Yes</td>
<td>4</td>
<td>0</td>
<td>1</td>
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<td>VZV No</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>9</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

HSV = herpes simplex virus; CMV = cytomegalovirus; VZV = varicella zoster virus
*Clinical and serological evidence of reactivation of viral disease after bone marrow transplantation

Cause of death (Table IV)

Four patients died due to leukemic relapse, two to acute GVHD, one to direct cardiopulmonary toxicity of the conditioning regimen, five to opportunistic infections which were related to chronic GVHD in three patients, and two to a clinical picture compatible with veno-occlusive disease. Thus, after a minimum follow-up of 1 year, eight patients survived. The quality of life for seven is good. One patient still suffers debilitating chronic GVHD.

Discussion

The present study provides evidence that intravenous administration of the rat monoclonal IgG2b antibody (Campath-1G) without additional immuno-

suppression after BMT permits engraftment and is associated with a low incidence of severe acute GVHD. The protocol was designed such that at the time of bone marrow infusion plasma levels of Campath-1G in the recipient were detectable so that effective elimination of donor lymphocytes was feasible. Since recipient lymphocytes disappeared within 24 h of the first Campath-1G administration, residual recipient cells that have been shown to be capable of surviving the conditioning regimen – thus leading to rejection of the graft,25 were likely to have been depleted from the recipient.

Acute GVHD can be prevented by in vitro depletion of T cells in donor bone marrow or administration of immunosuppressive drugs to the recipient after BMT.1 The extent of T cell depletion probably determines the incidence and severity of acute GVHD, 1 log depletion of T cells being associated with a considerably higher rate of acute GVHD than a 2 log depletion.26 In studies in which 2-3 log depletion was achieved there was approximately a 10% incidence of clinically significant acute GVHD, even when post-transplant immunosuppression was not administered. Relatively little information is available on the effect of T cell depletion on chronic GVHD, although the incidence

![Figure 1](image_url)  Concentration of Campath-1G in serum before conditioning and transplantation. Data are presented as means of measurements for 12 patients.
appears to be reduced in most studies. T cell depletion is complicated by an increased incidence of graft failure. From pooled data, it appears that the incidence of irreversible complete graft failure may be in the range of 10% for HLA-matched family donor transplantation. The most extensive experience with in vitro T cell depletion involves the rat IgM monoclonal antibody Campath-1M. A recent update of the results for several hundred allogeneic BMTs performed in the 'Campath-user groups' revealed an incidence of acute GVHD grade II-IV of 16% of the patients. Complete graft failure was observed in 15%.2,27

Severe acute GVHD (grade II-IV) was observed in only four of 21 evaluable patients (19%). This percentage is similar to that obtained with in vitro Campath-1M depletion but may be somewhat higher than the incidence reported for other in vivo T cell depletion procedures.1,28 The incidence of acute GVHD is also similar to that which can be achieved in BMT with post-transplant immunosuppression only.29 It is possible that the degree of T cell depletion in our patients was less than that achieved with some in vitro techniques resulting in a 2-3 log depletion.28 Thus, it is possible that the incidence of acute GVHD can be further decreased by prolongation of the Campath-1G infusion post-BMT or by increasing the dose.

In most reported series of patients who received HLA-identical sibling marrow grafts, the incidence of chronic GVHD was between 25 and 50%.30 For patients who had evidence of prior acute GVHD the probability of developing subsequent chronic GVHD was significantly higher than for those without or with only mild acute GVHD. In line with these data is the observation that patients who received T cell-depleted marrow developed chronic GVHD less frequently.31 Chronic GVHD is seen in only 18% of the patients who received a bone marrow graft that was depleted in vitro by Campath-1M.27 In our series the incidence of aGVHD does not appear to have been reduced (42%, 8/19 of the evaluable patients). In most cases, it was transient and responded to intensive treatment.

Immunological incompetence with the risk of opportunistic infections is apparent after BMT during the first 6-12 months. T cell function, as measured by proliferative response to phytohemagglutinin and normal IgG production, normalized 4-8 months after conventional BMT and 11-15 months after transplantation of T cell-depleted bone marrow.32-34 Grataua et al.21 reported reactivation of HSV infection in 45% and CMV infections in 47% of the patients after BMT. In our current series reactivation of viral diseases was observed in 19 episodes. This includes HSV reactivation in 40% and CMV reactivation in 47% of the patients at risk. Five patients died due to opportunistic viral, bacterial or fungal infections. It is conceivable that both the additional immunoincompetence caused by in vivo depletion of recipient and donor lymphocytes and the high incidence of chronic GVHD might be responsible for a number of these opportunistic infections. In this phase II study, however, the incidence of viral infections was not increased compared to the experience in previous years when systemic anti-lymphocytic antibodies were not used. The overall long-term survival for this group of patients was not particularly good, however, it has to be taken into account that a large proportion of these patients had recognized high risk features. No conclusion can be drawn regarding a specific diagnosis due to the small numbers.

In conclusion, in vivo depletion of recipient and donor lymphocytes with Campath-1G results in a relatively low incidence of acute GVHD without a concomitant increase in graft failure or relapse rate but with a relatively high incidence of chronic GVHD. A further decrease in the incidence of GVHD may be achievable by alteration of the infusion schedule or dose.

Acknowledgment

The authors wish to thank Clary Labec for preparing the manuscript.

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CAMPATH-1 Monoclonal Antibodies in Bone Marrow Transplantation

GEOFF HALE and HERMAN WALDMANN for CAMPATH USERS

ABSTRACT
CAMPATH-1 (CDw52) antibodies recognize a very small lipid-anchored glycoprotein that is expressed on the surface of human lymphocytes. They are remarkably lytic with human complement. In addition, CAMPATH-1G (rat IgG2a) and CAMPATH-1H (human IgG1) bind to human Fc receptors and are very effective for cell lysis in vivo. CAMPATH-1M (rat IgM) and CAMPATH-1G have been used to control GVHD and graft rejection in bone marrow transplantation by depletion of the T cells of the donor and recipient. Depletion of donor T cells alone gave excellent control of GVHD but up to 20% of the patients transplanted from HLA-matched siblings, and 51% of those transplanted from nonsibling donors, experienced graft failure caused by immunological rejection. Graft rejection could be partly overcome by additional immunosuppression either with CsA or total lymphoid irradiation (TLI). More effective was the use of CAMPATH-1G in vivo to deplete residual host lymphocytes. Preliminary results from current protocols of antibody depletion give two year actuarial leukemia-free survival as good as or better than similar studies with conventional GVHD prophylaxis, as well as a decreased morbidity from chronic GVHD, although engraftment was delayed by about 5 days. We propose that prophylactic T cell depletion with CAMPATH-1 antibodies is a simple and valid alternative to drug-based immunosuppression that may be particularly applicable to patients with acute leukemia or nonmalignant diseases transplanted from HLA-matched siblings as well as any patients transplanted from unrelated donors. Future developments of antibody-based immunosuppression may allow the extension of marrow transplantation for tolerance induction to organ transplants or in autoimmune diseases.

INTRODUCTION
Bone marrow transplantation can be used in the management of several different kinds of disease. It gives a remarkably powerful effect against some types of leukemia (notably CML) and would often be the treatment of choice for inborn or acquired defects of the hemopoietic system. In theory, it could be used to create tolerance to other organs and as a radical treatment for severe autoimmune diseases. The biggest problem is the two-way immune recognition of donor and recipient that leads to the major complications of graft-versus-host disease (GVHD) or graft rejection. With powerful immunosuppressive drugs, these can be controlled to some extent, yet GVHD still remains a significant cause of morbidity even when the donor and recipient are HLA-matched siblings. When the donor and recipient are less well matched, or unrelated, GVHD and graft rejection are major problems and this currently limits the use of marrow transplantation to only the most severe diseases. The key players in the generation of immune responses are T lymphocytes, and it has been shown that they are the prime mediators of

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GVHD and rejection. If T cell alloreactivity could be more effectively controlled, then the immunological complications could be overcome and marrow transplantation could be more widely applied.

Several years ago it was shown in animal models that removal of mature T cells from the donor bone marrow would prevent GVHD (1–4). However, ablation of recipient T cell function has not been so straightforward. Even after total body irradiation some recipient T cells remain and, in the absence of donor T cells, they can gain the upper hand and reject the marrow (5–7). The methods that were originally developed for depleting the donor T cells (e.g., agglutination with soybean lectin, E-rosette, density gradient centrifugation) were mostly not applicable to depletion of the same cells in vivo, so it was difficult to tackle this side of the problem with any specificity. The one type of agent that could be used both in vitro and in vivo was antilymphocyte globulin, and this was shown to be immunosuppressive and capable of preventing GVHD (8). The problem with antilymphocyte globulin is that it is a heterogeneous mixture of antibodies directed against different cell antigens, only a minority of which are T cell specific. It was therefore difficult to obtain samples that were reproducibly active and nontoxic.

When monoclonal antibodies became available, one of the first clinical applications was for purging marrow of donor T cells. However, most monoclonal antibodies were singly very inefficient at killing target cells because they did not activate human effector mechanisms (complement and/or Fc-receptor-dependent cell-mediated killing). Early efforts were therefore not very successful (9,10) and this led many investigators to search for different ways of using the antibodies for cell depletion. One method was to couple the antibody to a toxin (e.g., ricin) (11); others have used magnetic particles (12) or immunoadsorption (13). However, we continued to look for antibodies that could exploit natural human effector functions, with the prospect that these could eventually be used in vivo as well. Thus finding a lytic antibody for T cell depletion of bone marrow was not just an end in itself, but also a way to test reagents that might later find wider applications for immunosuppression.

THE CAMPATH-1 ANTIGEN

From a fusion of rat spleen cells with a rat myeloma cell line, we selected a set of antibodies that was unusually lytic for human lymphocytes using autologous complement (14–16). It turned out that all of them recognized the same antigen, which we now know to be a glycoprotein that is attached to the cell membrane by a lipid (glycosphingolipid) anchor that has a conventional structure similar to other mammalian GPI anchors. The N-linked carbohydrate, and indeed the 9 N-terminal amino acid residues, can be removed without destroying the antigenicity or sensitivity to complement lysis. The CAMPATH-1 antibodies therefore seem to recognize an epitope which includes the three or four C-terminal amino acids and possibly part of the GPI anchor. This implies that they bind very close to the cell membrane, which

surface antigens isolated from the same fusion, which were almost exclusively IgG\(_2a\), or IgG\(_{2b}\). The CAMPATH-1 antibodies recognized virtually all human lymphocytes, both T cells and B cells as well as monocytes and macrophages, although the latter seemed to be less sensitive to complement-mediated lysis. Tests on panels of fresh leukemia and lymphoma cells and cell lines showed that they recognized most cases of lymphoid malignancy, but only a small minority of myeloid leukemias (17,19). In the fourth leukocyte workshop the CAMPATH-1 antibodies were assigned to the cluster CDw52 (20). Little or no reactivity with colony-forming cells could be detected either by complement-mediated lysis, or by cell sorting (14,15). This important result was confirmed in numerous laboratories (21–24), although one group has reported only approx. 40% recovery of colony forming cells (25). Valentin and co-workers (26), using a different CDw52 antibody, which is known to have a broader reactivity than CAMPATH-1M, also found a significant depletion of CFU-GM. In these cases, we cannot be sure that the colony-forming cells did not require growth factors from the lymphocytes since the appropriate controls were not included. However, a recent comprehensive study of CAMPATH-1H found no evidence for reactivity with the cells that give rise to colony-forming cells during long-term culture (27).

CAMPATH-1 antibodies were found to recognize lymphocytes from old world monkeys, but not other species (15,23). At this time we also found that red cells were recognized in some of the monkeys. Antigen expression on the red cells seems to be under the control of a single autosomal gene, whereas expression on lymphocytes is constitutive (28). It has been reported that some CAMPATH-1 antibodies can recognize an antigen on human red cells (26), but despite extensive studies with large panels of cells using numerous techniques, we have never been able to detect such reactivity (15) (V. Taylor and G. Hace, unpublished observations).

Structure of the antigen

Recent work indicates that the antigen is a remarkably small glycoprotein, having only 12 amino acids, with a single N-linked carbohydrate on Asn-3, which is comparatively large and complex (Fig. 1) (18,29,30). At the C-terminus the peptide is attached to a glycosphingolipidinositol anchor that has a conventional structure similar to other mammalian GPI anchors. The N-linked carbohydrate, and indeed the 9 N-terminal amino acid residues, can be removed without destroying the antigenicity or sensitivity to complement lysis. The CAMPATH-1 antibodies therefore seem to recognize an epitope which includes the three or four C-terminal amino acids and possibly part of the GPI anchor. This implies that they bind very close to the cell membrane, which
Expression of antigen on spermatozoa

There is very little information about the physiological function of the CAMPATH-1 antigen. Like virtually all other GPI-anchored glycoproteins, there is some evidence that cells can be activated by CAMPATH-1 antibodies under suitable conditions (17,26,43). However, an intriguing observation was the discovery of the same antigen on mature human sperm (44–47). It actually seems to be synthesized by the epithelial cells of the epididymis, secreted into the seminal plasma (probably in the form of membrane-bound vesicles) from where it is taken up by the sperm as they pass through. Mature sperm are therefore sensitive to complement attack in the presence of CAMPATH-1 antibodies, but this is not thought to be a major problem in the context of therapy because only minute amounts of antibody would be expected to reach the seminal fluid and there the sperm would be protected by the large excess of vesicular antigen (47).

A homologous epididymal antigen has recently been identified in monkeys (48). It has a mature protein sequence of only 11 amino acids, which is very similar to the human antigen. It is likely that this is the same antigen that we detected on monkey lymphocytes. In mice, a related antigen has also been described that is expressed on lymphocytes and epididymis (49,50). Although the mature peptide (approx. 20 amino acids) does not closely match the human antigen, it contains a similar N-glycosylation site and the untranslated portions of the cDNA are significantly similar. We expect that these homologues will provide good targets for experiments to elucidate the antigen function. Meanwhile, they will also provide models for studying the therapeutic applications of CAMPATH-1 antibodies, which are by now well advanced in humans.

APPLICATIONS OF CAMPATH-1 ANTIBODIES IN BONE MARROW TRANSPLANTATION

CAMPATH-1M for T cell depletion

CAMPATH-1M was selected because it gave the highest titre of cell lysis with no effect on colony-forming cells. Studies in cynomolgus monkeys and in patients with end-stage lymphocytic leukemia showed no unexpected toxicity (51) and experiments in monkeys also showed no delay in the engraftment of autologous bone marrow treated with CAMPATH-1M and complement in vitro (52). On the basis of these studies, colleagues at the Hammersmith Hospital, London, the Hadassah University Hospital, Jerusalem, and the University Hospital, Ulm, started to use CAMPATH-1M with complement from donor serum for T cell depletion of donor bone.
marrow for allogeneic transplants. A series of 11 patients transplanted for advanced leukemia from HLA-matched siblings demonstrated that this was a very effective method of preventing GVHD, even when no posttransplant immunosuppression was used (21). Nevertheless, even in this small series, three cases of graft failure were observed, which we now know is the almost inevitable corollary of extensive T cell depletion.

Several lines of evidence suggest that the graft failure was mainly due to immunological rejection of the donor bone marrow, rather than, for example, an adverse effect of the antibody treatment on stem cells or their ability to repopulate the recipient (53). First, only one case of graft failure has been seen in more than 60 patients who received autologous bone marrow purged by the same method (to remove residual leukemia cells). Second, the incidence of graft failure was higher when bone marrow from unrelated or mismatched donors was used. Third, there were several cases in which host-derived cytotoxic T cells could be detected immediately preceding graft failure and some of them were shown to be specific for donor cells (54–56).

Since 1983, more than 500 transplants have been performed by 23 transplant centers throughout Europe and the Middle East, using this method of preventing GVHD in HLA-matched siblings (22,57–63) (Protocol 01, Fig. 2). Various modifications to the regime were made in an effort to reduce the risk of graft rejection. Some patients received posttransplant CsA. Others received intensified conditioning regimes, including additional radiotherapy, drugs, or total lymphoid irradiation.

A recent analysis (64) in patients transplanted for malignant diseases showed that the incidence of either severe or chronic GVHD was substantially reduced compared with other types of therapy. Overall, the frequency of acute GVHD > grade 1 in patients at risk was 17% and the frequency of severe acute GVHD (grade 3–4) was only 7%. The frequency of chronic GVHD was 19% and of severe chronic GVHD, 2%. The incidence of graft failure was lower in those patients who received CsA or TLI (12–13%) compared with those who did not (19–21% graft failure). The use of CsA (though not TLI) was also associated with a significant reduction in transplant-related mortality. Only a small number of patients (n = 24) received both TLI and CsA, but they fared particularly well with only 4% graft failure.

Prevention of rejection with Campath-1G

The partial success in prevention of graft failure by immunosuppressive reagents led us to believe that better control might be achieved with more selective and potent immunosuppressants. Experiments in animals showed that treatment of the recipient with depleting anti-T cell antibodies could abolish rejection of T cell-depleted bone marrow, even with reduced irradiation or when the donor and recipient differed across major histocompatibility barriers (7,65). We therefore looked for monoclonal antibodies that could produce a similar effect in humans. Antibodies that could deplete lymphocytes in vivo were important in these experimental studies and so again we turned to the Campath-1 antibodies since they were the most effective reagents available at the time (66).

The original IgM antibody Campath-1M, although extremely lytic with complement, gave only transient depletion of lymphocytes in vivo in patients with CLL or lymphoma (51). However, from another CDw52 clone that secreted an IgG2a antibody, we were able to isolate a spontaneous class-switch variant, the rat IgG2b antibody Campath-1G (62). The IgG2b subclass is not only the best rat IgG for activating complement but is also optimal for binding to human Fc receptors and activating cell-mediated killing (67–69). Campath-1G proved to be very efficient at depleting lymphocytes in vivo in several patients with lymphoid malignancies and in patients

<table>
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<th>1 Original (many)</th>
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<td>BM/Campath-1M+C'</td>
</tr>
<tr>
<td></td>
<td>Campath-1G--</td>
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<tr>
<td>3 In the bag (JER/CAP)</td>
<td>BM/Campath-1G</td>
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<tr>
<td>4 In vivo (LDN)</td>
<td>BM</td>
</tr>
<tr>
<td></td>
<td>Campath-1G--</td>
</tr>
<tr>
<td>5 In vivo (HAM)</td>
<td>BM</td>
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<td></td>
<td>Campath-1G--</td>
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<td>6 In vivo (consensus)</td>
<td>BM</td>
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<td></td>
<td>Campath-1G</td>
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<tr>
<td>7 In vivo plus in the bag</td>
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Day | -15 | -10 | -5 | 0 | 5

FIG. 2. Illustration of the different antibody protocols studied by the Campath users group. The dose of Campath-1M was typically 25 mg for in vitro purging, whereas the dose of Campath-1G was usually 5–10 mg for in vitro purging or 5–10 days at 5–10 mg/day for in vivo treatment.
suffering steroid-resistant kidney graft rejection (70,71). In both studies, the treatment with CAMPATH-1G gave a beneficial clinical response in many of the patients.

The first objective was to see whether treatment with CAMPATH-1G would decrease the risk of graft rejection in the context of "standard" T cell depletion. This was not easy to test because the actual incidence was comparatively low, and so quite a large number of patients was needed to demonstrate an effect. Initially we did not know whether the recipient's effector mechanisms would be compromised by the conditioning regime, so it was possible that administration of antibody at the time of the transplant might not be effective. Therefore the CAMPATH-1G was given at 5–10 mg/day for 5 days before the start of chemoradiotherapy (Protocol 02). Although this had the slight disadvantage that extra inpatient time was required, the protocol had several advantages: (a) the degree of lymphocytopenia produced by CAMPATH-1G could be measured, uncomplicated by other treatments, (b) adverse effects of the antibody could likewise be assessed, and (c) most of the antibody would be cleared by the time of the transplant and so be unlikely to contribute to extra depletion of the donor T cells. T cell depletion with CAMPATH-1M and other elements of the conditioning regime were kept the same as in historical control groups. No other prophylactic agents to prevent GVHD or graft rejection (e.g., CsA, TLI) were given. The plan is to enroll 100 patients with acute leukemia in first remission, which will provide a homogeneous group where the effect of the antibody protocol on relapse can also be observed.

To date about 75 such patients have been enrolled by three transplant teams (Royal Free, London; University Hospital, Ulm; King Faisal Hospital, Riyadh). The interim results are very satisfactory and a detailed report will be published when the study is complete. Meanwhile we have analyzed these study patients as part of a larger group of patients, including CML and advanced disease, who all received the same antibody protocol (Table 1). Out of 144 patients there has been only 2% severe acute GVHD, no case of severe chronic GVHD, and 9% graft failure. The risk of death at 2 years from all causes other than relapse is 21 ± 8%, which is significantly lower than that seen with the original protocol (28 ± 3%). Furthermore, the leukaemia-free survival at 2 years is also significantly better (Fig. 3A). Although graft failure has not been totally eliminated, there is a possibility that in the future, the addition of a short course of CsA might make the risk still lower.

The degree of lymphocyte clearance induced by CAMPATH-1G in vivo has been measured by a sensitive limiting dilution analysis and found to be similar to that achieved with busulphan/cyclophosphamide or total body irradiation (72). Antibody therapy was as effective as the conventional treatments at depletion of cytotoxic T cell precursors (> 99%) but far more effective at eliminating helper T cells that were relatively resistant to chemotherapy or radiotherapy.

The use of CAMPATH-1G for prevention of GVHD

The successful use of CAMPATH-1G to eliminate cells in vivo suggested that it could be used in a similar way to remove the donor T cells in order to prevent GVHD. In principle, this could be done either by addition of the antibody to the donor bone marrow to opsonize the cells for subsequent clearance in vivo (protocol 03), or by administration to the patient at or around the time of the transplant (protocols 04–06). In both cases, the antibody should also help to remove residual host lymphocytes though its effect on the donor T cells would presumably be greater when it was admixed with the bone marrow. The advantage of these procedures is that no additional complement or processing of the bone marrow would be required. Both approaches have been tried by different transplant centers and the results prove that sufficient depletion of donor T cells to avoid GVHD can be achieved by either method (Table 1).

Teams at the Hadassah Hospital, Jerusalem and the University Hospital, Cape Town have used CAMPATH-1G in vitro (Protocol 03) (73–75). Most patients (24/26) received additional total lymphoid irradiation to prevent graft rejection, but no posttransplant immunosuppression. The incidence of either severe GVHD or graft failure was very low, but as the number of patients so far treated is small, this is not yet significantly different from the results with the original protocol. However, the 2 year transplant-related mortality of this group of patients is already significantly better than those treated on the original protocol and so is the leukemia-free survival (Fig. 3B).

These results provide convincing evidence that mere opsonization with CAMPATH-1G is sufficient to deplete the donor T cells. We know that CAMPATH-1G can bind to human Fc receptors as well as activating human complement, but we cannot say which mechanism is more important. If the donor bone marrow was not washed free of plasma, significant lysis of T cells was obtained within 30 min of addition of CAMPATH-1G, even though no extra complement was added (W. Fibbe and R. Willemze, University Hospital, Leiden and E. Yousaf and D. Pamphilon, Blood Transfusion Service, Bristol, unpublished observations). However, the procedure seemed to be equally as successful with bone marrow separated on Ficoll and washed free from plasma, so presumably the necessary effector mechanisms are sufficiently intact in the recipient despite the chemoradiotherapy.

An alternative way of using CAMPATH-1G to prevent GVHD has been tested by teams at the University Hospi-
### Table 1. Results in Matched Sibling Transplants for Malignant Disease

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<thead>
<tr>
<th>Protocol</th>
<th>No.</th>
<th>In vivo</th>
<th>In vitro</th>
<th>No. of patients</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
<th>Either</th>
<th>Actuarial at 2 years ± SE</th>
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*The fraction who suffered graft failure or GVHD is calculated as a percentage of the patients at risk. Patients were considered not evaluable for engraftment if they died before day 20 without engraftment, and not evaluable for GVHD if they suffered graft failure or died before day 100 without GVHD (day 120 for chronic GVHD). Results in each protocol were compared with the original protocol (01) by the χ² test. Those that are significantly better are in boldface and those that are worse are in italics. Note: Leukemia-free survival means freedom from hematologic disease. Cytogenetic relapse in CML was not included.*
PATH-1M \textit{in vitro}. However, an observation that may be relevant is that all of the patients who have received CAMPATH-1G \textit{in vivo} had slower engraftment than normal, with a median time to reach 500 neutrophils of about 24 days compared with a median of about 19 days for patients who had only \textit{in vitro} T cell depletion (64). This delay in engraftment was more marked when the CAMPATH-1G was given close to or around the time of the transplant. We do not think there was a direct toxic effect on bone marrow stem cells because no delay was seen with CAMPATH-1G \textit{in vitro}. Possibly, a small number of lymphocytes, either donor or host, play a role in engraftment, perhaps by secreting cytokines that promote the growth of progenitor cells.

\textbf{Leukemia relapse}

One of the potential risks of T cell depletion for patients with leukemia is the loss of a beneficial graft-versus-leukemia effect. In 1979 the Seattle transplant team showed that patients with advanced acute leukemia who suffered from GVHD were at lower risk of relapse, compared with those who did not develop GVHD (78). Subsequent analysis of large collections of data has confirmed this finding in all the major categories of leukemia treated by marrow transplantation (79). There has been considerable speculation as to whether the beneficial graft-versus-leukemia effect can be separated from GVHD, perhaps by selective manipulation of the donor T cells, and there have been several experimental studies in animals which suggest that this may be possible (80–83). However, there are very considerable practical difficulties in applying these principles to human therapy. At present, we can only examine whether the trade-off between decreased transplant-related mortality and increased relapse is favorable or not.

The risk of relapse for all patients with early leukemia who received T cell-depleted transplants is shown in Fig. 4. Results of both \textit{in vitro} and \textit{in vivo} depletion are included, but patients who received any form of T cell addback are excluded. With up to 10 years follow-up, the risk of relapse in ALL and AML shows a plateau at approx. 30%, with most cases occurring in the first year. In CML, the risk of hematologic relapse continues to rise for at least 5 years, reaching an apparent plateau of about 60% by 7 years. It has been clear for some time that T cell depletion results in a significant increase in relapse risk for patients with CML transplanted from matched siblings (59,61,62,84,85), though it was initially surprising to find that the risk of relapse of T-depleted patients is much greater than for conventionally treated patients who do not suffer from GVHD (79). This is perhaps the best evidence yet in humans that the antileukemic effect of the marrow transplant might be distinct from clinical GVHD.
In contrast, the results in acute leukemia do not show such a distinction, since the effect of T cell depletion can be accounted for in terms of the improved control of GVHD. The relapse rates we see are a little greater than reported using conventional therapy with MTX/CsA (86,87) and this results in a slightly lower leukemia-free survival when we consider the CAMPATH-1-treated patients as a whole. However, the overall control of transplant-related complications is so much better with the recent protocols, particularly 02 and 03, that this should compensate for the slightly increased risk of relapse. Currently the leukemia-free survival at 2 years for patients with acute leukemia treated according to protocol 02 or 03 is 56%, which is almost exactly what would be expected using MTX/CsA. However, the improved control of GVHD, particularly chronic GVHD, with antibody therapy is likely to be a significant advantage.

Transplants for nonmalignant diseases

If antibodies could be routinely used to control GVHD and rejection, one of their most useful applications would be in transplants for nonmalignant diseases, such as severe aplastic anemia and eventually thalassemia or sickle cell anemia. These situations are not complicated by the potential graft-versus-leukemia effect of donor T cells. Irradiation is also undesirable and so there would be significant long-term advantages in obtaining a powerful but nontoxic immunosuppressive conditioning regimen.

To date the CAMPATH-1 users group have carried out 116 such transplants from HLA-matched siblings (80 aplastic anemia, 22 thalassemia, 9 Fanconi’s anemia, 5 other inborn errors). A diversity of conditioning regimens and antibody treatment protocols has been tested as in the patients with malignant diseases. However, the numbers of patients treated according to each individual protocol are small. Two main groups of patients can be identified according to the antibody treatment (Table 2). The first group received T cell depletion in vitro, either with CAMPATH-1M (31 patients, protocol 01) or CAMPATH-1G (16 patients, 17 transplants, protocol 03). The second group received CAMPATH-1G in vivo either with

<table>
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<tr>
<th>Protocol</th>
<th>No. of patients</th>
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<th>Chronic GVHD</th>
<th>Actuarial at 2 years ± SE survival</th>
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\[ \text{Percentages} \]
T cell depletion in vitro (11 patients, protocols 02, 07) or without (9 patients, protocol 06). Three of the transplants in the second group were retransplants after graft rejection of patients in the first group (75). In every case the control of GVHD has been excellent. There were only two cases of acute GVHD > grade 1 and only one case of chronic GVHD (not severe). In the group who received only in vitro T cell depletion, the incidence of graft failure was 45%, but in the group who received in vivo T cell depletion it was only 5% (p < 0.01, χ² test). The actuarial survival at 1 year for the in vitro group was 74% and for the in vivo group 100% (p < 0.03, log-rank test). Unlike the situation in transplants for leukemia, the use of CAMPATH-1G in vivo was not associated with any delay in engraftment. The median day to reach 500 neutrophils for the in vitro group was day 19 and for the in vivo group was day 14 (excluding patients who did not engraft). Unlike the patients with leukemia, most of these patients did not receive TBI. Possibly their bone marrow microenvironment was therefore more conducive to rapid regeneration.

Transplants from donors other than HLA-identical siblings

Only a minority of patients who might benefit from a marrow transplant have a suitable HLA-identical sibling. Patients who receive transplants from HLA nonidentical family members or from "HLA-matched" unrelated donors are at significantly higher risk of GVHD and other severe transplant complications. Therefore much attention has been given to the use of T cell depletion in these situations. CAMPATH-1 antibodies have been particularly used in transplants from parents for immune deficiencies and other inborn errors (88–92) and in unrelated donor transplants (93–95).

A summary of the results to date is given in Table 3. T cell depletion in vitro gave good control of GVHD with only 25 cases of severe acute GVHD out of 180 patients evaluable (14%). However, the incidence of graft failure was substantial (51%) in those patients who did not receive additional therapy with CAMPATH-1G in vivo. This is despite the fact that many of them received various other treatments designed to reduce the risk of graft rejection, such as additional irradiation or CsA or other antibodies, including LFA-1 and CD2 (92). Patients who received in vitro T cell depletion combined with in vivo treatment with CAMPATH-1G had a significantly lower incidence of graft failure (24%), though it was still a substantial problem. The use of in vivo antibody alone combined with conventional GVHD prophylaxis using MTX/CsA gave a similar rate of graft failure and GVHD.

Because these results included all categories of patients with early or advanced leukemia or nonmalignant disease, it would be uninformative to compare the survival curves. The largest subset is the patients transplanted for CML in chronic phase from unrelated donors (94). They are of particular interest in view of the high incidence of relapse that was seen following T cell depletion in HLA-matched sibling transplants. The patients are divided into two groups (Table 4). Thirty patients received in vivo CAMPATH-1G combined with in vitro T cell depletion (Protocol 02) and 60 received only in vivo CAMPATH-1G with conventional GVHD prophylaxis (Protocol 05/06). There were no significant differences between the incidences of graft failure and GVHD in the two groups although, as expected, the trend was toward less GVHD but more graft failure with in vitro T cell depletion. However, in both cases the incidence of relapse seemed to be much lower than in comparable transplants from HLA-matched siblings (Fig. 5). Two-year survival was significantly better in the group who received only in vivo antibody, and the

<table>
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<th>Protocol</th>
<th>No. of patients</th>
<th>Graft fail</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
<th>Actuarial at 2 years ± survival</th>
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</tr>
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<td>11</td>
<td>13</td>
<td>4</td>
<td>55</td>
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<td>48 ± 14</td>
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leukemia-free survival in this group \((53 \pm 15\%)\) was as good as that obtained in matched sibling transplants and superior to published results for unrelated donor transplants using conventional GVHD prophylaxis \((96)\).

**Lymphoproliferative disease, immune reconstitution, and infections posttransplant**

Unlike some other trials of T cell depletion \((97-100)\) lymphoproliferative disease was rare. Out of 1529 transplants, 10 cases of lymphoproliferative syndrome or secondary lymphoma have been reported \((101)\). This represents an actuarial risk at 5 years of 1% in transplants from siblings \((5/1067)\) and 2.3% from mismatched or unrelated donors \((n = 5/462)\). CAMPATH-1 antibodies recognize B cells as well as T cells and it is possible that bone marrow purging with CAMPATH-1M or CAMPATH-1G helps to reduce the potential target for EBV-driven oligoclonal proliferation of B cells in the early posttransplant period, which can lead to fatal malignant lymphomas if uncontrolled.

The CAMPATH users group have not centrally collected data on the recovery of lymphocyte subsets or the incidence of nonfetal infections because of the huge amount of complex reporting and analysis this would have entailed. The number of fatal infections late after transplant did not seem to be more than seen with conventional GVHD prophylaxis, since transplant-related mortality did not increase much after the first 9 months. However, individual centers have observed relatively slow T cell regeneration following lymphocyte depletion with CAMPATH-1M \((102,103)\) and some have found a high incidence of CMV viremia \((A\ Bacigalupo, Genoa, and D. Bunjes, Ulm, unpublished observations)\). Since the normalizations of T cell numbers may not occur for a year or more after a bone marrow transplant even with conventional GVHD prophylaxis, it is not easy to assess the impact of T cell depletion on this parameter. Furthermore, we do not know how well the T cell phenotype really reflects the immune status of an individual. Probably functional assays of T cells are more relevant although they are more laborious to carry out.

**CONCLUSION**

T cell depletion is certainly the most effective method of preventing graft-versus-host disease. We have developed simple procedures to accomplish this using the patient's own immune effector mechanisms. CAMPATH-1 monoclonal antibodies are particularly effective, possibly because of the unique properties of their target antigen. Furthermore, there is the possibility that in vivo antibody treatment may contribute to elimination of residual leukaemia in some cases \((70)\).

Better control of GVHD is a prerequisite before bone marrow transplantation can be fully used in the treatment of a wide range of diseases. Unfortunately, T cell depletion is associated with increases in the risks of graft rejection and leukemia relapse. Substantial progress has been made toward overcoming the problem of graft rejection by giving increased immunosuppression to the recipient, either with CsA or TLI, or most successfully by in vivo treatment with CAMPATH-1G. Eventually we believe that a combined strategy should permit successful engraftment in virtually all HLA-matched sibling transplants and the great majority of others. This should mean that bone marrow transplants can be used for nonmalignant hematological diseases with less fear of procedure-related mortality and it might open the way to the use of marrow transplantation for tolerance induction in organ transplantation and autoimmune disease.

The risks of leukemia relapse associated with extensive T cell depletion have now been well defined, at least in patients with early disease. In acute leukemia the increase in risk is modest compared with current results using drug-based GVHD prophylaxis. The extra mortality from relapse is likely to be offset by a reduction in transplant-related complications. In CML, T cell depletion has a significant adverse effect, at least in patients transplanted from HLA-matched siblings. We do not know why a similar increase in relapse was not seen in patients transplanted from unrelated donors. It may be relevant that most of the latter group receive higher doses of radiotherapy \((95)\), but it is also possible that there is a stronger allogegenic effect that is not completely abrogated by the antibody depletion.

Some groups have tried to add back small numbers of lymphocytes to the depleted bone marrow or after the transplant in order to preserve a graft-versus-leukemia effect \((74,104,105)\). The effect has been to increase the risk of GVHD and decrease the risk of leukemia relapse.
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(64) and, overall, the results are similar to those obtained without T cell depletion. In other cases donor T cell infusions have been used to effectively treat relapse (106, 107), but this procedure is not always effective and carries a high risk of fatal GVHD. Ideally, T cell addback could be administered 2–4 months after the transplant when the patient had recovered from the effects of the conditioning regime but before hematological relapse is likely to have occurred. If a sensitive assay were available to indicate the amount of residual disease at that stage (108), this immunotherapy could then be reserved for only those patients who were likely to relapse and had not suffered any GVHD. However, whatever refinements are made in the timing, dose, or cellular content of lymphocyte therapy, it is unlikely that its potential for producing severe GVHD will be eliminated. The biological effect will largely be dictated by the immune repertoire of the lymphocytes and the genetic disparity between donor and recipient. The alternative strategy is to seek for new drug treatments for residual leukemia, or more effective combinations of existing ones. For example, the drug thiotalpa is reported to be myelosuppressive and may be useful for treatment of CML in the context of T cell depletion (109, 110). So far the clinical results are very early, and longer follow-up is needed to be sure whether this treatment will give improved results.

Bone marrow transplantation will continue to be an important arena for the testing of new types of biological therapy. As the clinical results gradually improve and the range of potential new treatments becomes broader, it will be more and more difficult to design prospective trials that have a good chance of showing significant improvements. We believe that the best use can be made of these limited opportunities if we test therapies that, like CAMPATH-1, potentially have a very broad application. Although we have not described here the wider range of clinical applications where CAMPATH-1 antibodies are being used, such as for treatment of leukemia, control of organ transplant rejection, and treatment of autoimmune diseases (70, 71, 111–114), nevertheless, the results obtained from the studies in bone marrow transplantation have helped to make those possible.

ACKNOWLEDGMENTS

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T-cell-depleted allogeneic bone marrow transplantation for acute leukaemia using Campath-1 antibodies and post-transplant administration of donor’s peripheral blood lymphocytes for prevention of relapse

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Summary. One hundred and forty-six patients with acute leukaemia (81 with ANL and 65 with ALL) received allogeneic bone marrow transplantation from their fully matched siblings. 121 patients underwent T-cell depletion (TCD) using Campath 1 monoclonal rat anti-human lymphocyte (CDw52) antibodies; 67 with Campath 1M and 54 with Campath 1G isotypes. Patients were conditioned for transplant using either total body irradiation combined with chemotherapy (125 patients) or busulfan and cyclophosphamide (21 patients). 112 recipients of T-cell depleted allografts received in addition total lymphoid irradiation (TLI) for prevention of rejection. Engraftment of neutrophils (>0.5 × 10⁹/l) and platelets (>25 × 10⁹/l) occurred on days 15 and 18, and on days 18 and 20 in recipients of Campath 1M and Campath 1G treated marrows respectively. Rejection was documented in 6-8% of T-cell depleted transplants. Leukaemia relapse-free survival at 2 years was 83% for patients transplanted in first CR, 76% in second CR (P² = 0.34) and 42% in advanced leukaemia (P² = 0.009).

81 marrow recipients, 38 with Campath 1M and 43 with Campath 1G treated marrow, received post-transplant graded increments of donor’s peripheral blood lymphocytes (PBL) to induce graft-versus-leukaemia (GVL) effects. Administration of donor’s PBL was associated with clinically significant GVHD and with decreased relapse rate especially in patients with ALL. Our data suggest that in patients receiving marrow allografts depleted of T cells by Campath 1 monoclonal antibodies, rejection can be reduced by adequate pregrafting immunosuppression. In patients with advanced disease, post-transplant cell-mediated immunotherapy (CMI) using donor’s PBL may be beneficial; however, further studies are needed to define the optimal schedule of CMI for safe and effective prevention of relapse following TCD bone marrow transplantation in malignant haematological diseases.

Keywords: allogeneic BMT, T-cell depletion, donor lymphocytes.

Acute and chronic graft-versus-host disease (GVHD) is the primary complication of allogeneic BMT affecting one-third to a half of HLA-matched bone marrow transplant recipients with potentially severe multisystemic complications which may lead to poor quality of life with occasional serious or even fatal outcome (Sullivan, 1986; Gale et al. 1987; Atkinson et al. 1990).

The most successful prophylaxis against GVHD consists of combined administration of ciclosporin A and methotrexate (Storb et al. 1986, 1988; Deeg & Henslee-Downey, 1990), a combination associated with delayed engraftment and increased rate of leukaemic relapse (Storb et al. 1989; Sullivan et al. 1989a). Alternatively, effective prevention of GVHD may be accomplished by pretransplant depletion of...
Donor PBL for Prevention of Relapse in T-cell-depleted BMT

Donor immuno-competent T cells (T-cell depletion, TCD) (Waldmann et al. 1984; Martin et al. 1985; Slavin et al. 1985; Frame et al. 1989; Antin et al. 1991). T-cell depleted marrow allografts are, however, susceptible to rejection (Keroan et al. 1989; Bordignon et al. 1989), which may be prevented by more intense immunosuppression of the host during the pre-transplant conditioning (Slavin et al. 1986). Moreover, effective depletion of lymphocytes from the marrow prevents the immune-mediated interaction of donor T cells against residual host tumour cells – the graft-versus-leukaemia (GVL) phenomenon (Sullivan et al. 1989a; b; Martin et al. 1985; Gale & Reimer, 1986; Weiden et al. 1979, 1981; Horowitz et al. 1990). Therefore the relapse rate in chronic myeloid leukaemia, and to a lesser extent in acute leukaemia, is significantly higher following TCD when compared with non-T-cell depleted allografts (MacKinnon et al. 1990; Truitt & Atasoy, 1991; Goldman et al. 1988). Because it has been shown consistently that GVHD induced by T cells is associated with GVL effects, it would therefore seem desirable to retain their antileukaemic effects and at the same time prevent or control the GVHD associated with their administration. We have shown in an experimental animal model of murine B cell leukaemia/lymphoma (BCL1) that delayed administration of allogeneic immunocompetent T cells after TCD transplant can effectively induce GVL without GVHD (Weiss et al. 1992; Slavin et al. 1978, 1992). Similar observations were recently reported in patients relapsing after BMT (Drebycki et al. 1992; Kohl et al. 1990; Porter et al. 1994). We have therefore chosen to use a similar approach by gradual transfer of controlled increments of donor's peripheral blood T lymphocytes (PBL) in order to reduce the risk of leukaemic relapse in recipients of TCD marrow allografts (Slavin et al. 1988).

Data presented in this report summarizes our initial results in allogeneic BMT for acute leukaemia, using Campath (monoclonal rat and human CD52) antibodies for TCD and post-transplant cell-mediated immunotherapy (CMI) with donor PBLs.

MATERIALS AND METHODS

Patients. Between 1981 and 1991, 146 patients with acute leukaemia were referred to the Bone Marrow Transplantation Centre at the Hadassah Hospital in Jerusalem for allogeneic BMT. All had HLA-identical, MLC non-responding sibling donors. Patients were eligible for transplantation irrespective of the stage of leukaemia if there was no life-threatening associated disease or organ impairment. Patient stratification into types of leukaemia and stage of disease is summarized in Table 1. Patients in third or subsequent remission, non-responsive or resistant relapse, or patients with extramedullary disease, were categorized as advanced stage.

All the treatment protocols were approved by the Hadassah Hospital IRB and the Ministry of Health Human Experimentation Committee. Written informed consent was obtained from all adult patients and donors, and court permission was obtained in each case of a minor donor.

Pretransplant conditioning. All patients with ALL were treated with total body irradiation (TBI) and chemotherapy, whereas patients with ANLL were treated either with busulfan and cyclophosphamide (BU-CY) or with combined

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<th>ALL</th>
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1 Busulfan 50 mg/kg/d x 4 and cyclophosphamide 50 mg/kg/d x 4.
2 Patients received in addition total lymphoid irradiation (TLI).
3 TBI 200 cGy x 6 and cyclophosphamide 60 mg/kg/d x 2.
4 Same as 3 but with addition of melphalan 60 mg/m² x 1 or cytosar 500 mg/m² x 4.
5 TBI 200 cGy x 6, cyclophosphamide 50 mg/kg, melphalan 60 mg/m², and VP-16 1500 mg/m².
chemoradiotherapy (Table I). Since July 1984, 114 patients receiving TCD allografts were given additional total lymphoid irradiation (TLI) for further immunosuppression and prevention of rejection (Slavin et al., 1986).

**Chemotherapy.** 21 patients were treated with BU-CY (busulfan 4 mg/kg/d for 4 d and cyclophosphamide at 50 mg/kg/d for 4 d). 117 patients were conditioned with TBI and chemotherapy. Over the years, three different regimens were used: during 1981–85 a single drug, consisting of cyclophosphamide 60 mg/kg/d for 2 d (38 patients), during 1986 a two-drug combination consisting of similar doses of cyclophosphamide and melphalan (60 mg/m² × 1) or cytosar (500 mg/m² × 4) (13 patients), and since 1987 a triple chemotherapeutic combination consisting of etoposide (VP-16) 1500 mg/m² × 1, cyclophosphamide 60 mg/kg × 1 and melphalan 60 mg/m² × 1 (66 patients). The remaining eight patients were treated with modified conditioning regimens according to individual needs. Unless contraindicated, all patients received two intrathecal doses of methotrexate and cytosar before the transplant.

**Irradiation.** TBI was administered in six fractions of 200 cGy each every 12 h, at 20 cGy/min over 3 d to a total of 1200 cGy. Partial lung shielding was accomplished by half value leaf shield after 600 cGy. TLI was administered at 80–120 cGy/min in four fractions of 150 cGy each over 2 d to a total of 600 cGy for patients scheduled for TBI, and five daily fractions of 200 cGy to a total of 1000 cGy for patients scheduled for BU-CY. Whenever indicated, patients with ALL received supplemental cranial and testicular irradiation.

**Bone marrow transplantation and T-cell depletion (TCD).** Bone marrow was harvested under epidural (adult donors) or general (minor donors) anaesthesia. 15 patients received unmanipulated marrow allograft (13 of them underwent BM transplant during 1981) and received standard GVHD prophylaxis consisting of methotrexate alone.

One hundred and thirty-one patients received TCD marrow allografts: 10 patients using the sheep red blood cell rosette (E rosette) separation technique with or without soybean agglutination (SBA) (Reimer et al., 1984), and, since July 1983, using monoclonal rat anti human CDw52 (Campath-1) antibodies as previously described (Hale et al., 1983; Waldman et al., 1984; Slavin et al., 1985).

**TCD using Campath-1 mAb. Campath 1M (IgM kappa).** Marrow buffy coat fraction was prepared by sedimentation with hetastarch (0-66%). Cells were resuspended in Hanks balanced salt solution at a concentration of 70-100 × 10⁶ cells/ml, and Campath 1M added at 0-1 mg/10⁶ cells for 30 min at room temperature. Fresh donor serum (20%) was then added as a source of complement for 30 min at 37°C. Cell clumps were carefully removed and the marrow was washed and infused (Hale et al., 1983; Cobbold et al., 1986).

**Campath 1G (IgG2b kappa).** Campath 1G was added directly into the marrow bag at three different concentrations: 0-3 (17 patients), 1 (30 patients) and 3 mg/10⁶ nucleated marrow cells (7 patients). Marrow cells were infused following 30 min incubation at room temperature and gentle agitation (Naparstek et al., 1989). In ABO mismatched transplants, RBC were first removed by sedimentation with hetastarch and Campath 1G was added into the buffy coat.

Residual T lymphocytes in marrow treated with Campath 1M were estimated consistently through the years by E-rosettes.

**Engraftment and chimerism.** The day of marrow infusion was designated as day 0. Engraftment was defined as an ANC of >0-5 × 10⁹/l for three consecutive days and unsupported platelets >25 × 10⁹/l. Engraftment was confirmed whenever possible by donor's cytogenetic markers and by red blood cell antigens and isoenzymes.

**Post-transplant cell-mediated immunotherapy (CMI) using donor peripheral blood lymphocytes (PBL).** 81 patients received fresh unirradiated donor's PBLs at weekly intervals for induction of graft-versus-leukemia (GVL) activity. The lymphocytes were collected by venipuncture or by continuous-flow apheresis using Barter's CS 3000 plus. Cell dose was calculated as the equivalent proportion of T cells/kg.

Two regimens for graded post-transplant T-cell repletion (TCR) were applied: (1) patients transplanted with Campath 1M treated marrow and patients receiving marrow treated with high concentrations (3 mg/10⁶ cells) of Campath 1G received donor's T cells early, beginning on day 1+1 with 10⁴ or 10⁵ T cells/kg, followed by a general schedule of weekly 1 log increment for a total of up to four doses (10⁴, 10⁵, 10⁶, and 10⁷ T cells/kg on days 1+1, +6, +14, and +21 or +28 respectively); (2) patients transplanted with marrow treated with low concentration of Campath 1G received donor's T cells only if no signs of acute GVHD developed by day 28; thereafter they received donor's PBL at an equivalent proportions of 10⁵, 10⁶, 10⁷ T cells/kg on days +28, +56 and +84, respectively. Subsequent administration of donor's PBL was discontinued as soon as signs or symptoms suggestive of acute GVHD appeared.

**Statistical evaluation.** Results were analysed as of August 1991. The follow-up period extended from 10 years to 1-5 months for the most recent patient included in the study, with median potential follow-up of 3-7 years.

Event-free survival (either death in remission or relapse) and relapse-free survival were computed from the date of transplant until date of death or relapse, or were censored at appropriate dates if these events had not occurred. Patients who died without relapse were considered as censored observations in relapse-free survival analyses, whereas the first event experienced by a patient (relapse or death without relapse) was considered a failure for event-free survival. The Kaplan-Meier method was used to calculate the probability of event-free survival or relapse-free survival as a function of time (Kaplan & Meier, 1958). The significance of the differences between pairs of Kaplan-Meier curves was determined by the Mantel-Haenszel technique (Mantel, 1966). Cox proportional hazards modelling (Cox, 1972) was used to identify whether any factors are jointly predictive of the probability of event-free or relapse-free survival. Patient characteristics considered for evaluation in the models included type of leukaemia and stage of disease at the time of transplantation, age, pretransplant conditioning, method of T-cell depletion, presence of acute or chronic GVHD and T-cell repletion. All P values are two-sided and denoted by P².
RESULTS

Patient characteristics and pretransplant condition
One hundred and forty-six consecutive patients with acute leukaemia, 82 with ANLL (among them 11 patients with MDS and four with secondary leukaemia) and 64 with ALL (among them three patients with leukaemic relapse of lymphoma), received marrow allografts from fully matched sibling donors. Table I summarizes the patients characteristics at the time of transplantation. The majority of patients with ANLL (61%) were in first CR, whereas 73% of patients with ALL were in second or subsequent remission. Overall, 29% of the patients in both disease categories were transplanted in advanced disease, among them 19 in second or unresponsive relapse. The median age of patients transplanted for ALL was significantly lower than that of ANLL patients (17 and 24 years, p = 0.00312), particularly among those transplanted in advanced stage. 122 patients received TBI combined with single, double or triple chemotherapy, whereas 21 patients, mostly children (median age 13 years) were conditioned with BU-CY, all of them with ANLL 17 in first CR.

Bone marrow transplantation, T-cell depletion and engraftment
Table II presents data on the methods of T-cell depletion employed. Similar numbers of patients in each disease category received marrow treated with either Campath 1M or Campath 1G. The number of bone marrow cells infused varied according to the method of purging and in vitro manipulation. In 32 patients receiving marrow treated with Campath 1M, residual T lymphocytes could not be detected, and in the remaining patients the mean percentage of T cells infused was 0.08%. In Campath 1G treated marrow, estimation of the number of infused T lymphocytes is somewhat more difficult, because T-cell depletion is expected to occur in vivo, most likely through antibody-dependent cell-mediated cytotoxicity (ADCC).

Uneventful engraftment occurred in 126 patients; two additional patients had prolonged pancytopenia, received a top-up marrow and reached satisfactory blood counts with documented donor type cells. The median day to attain ANC > 0.5 x 10^9/L was 15.5 d and to reach unsupported platelets > 25 x 10^9/L was 16 d for Campath 1M treated marrow recipients and 18 and 20 d respectively for those receiving marrow treated with Campath 1G.

Nine patients (6%) died within the first 18 d (median 14 d) before attaining sustained engraftment. Causes of death were infection, hepatic failure, ARDS and bleeding.

Rejection and graft failure
Nine recipients of T-depleted marrow (Table II) rejected their allografts (6-8%). Initial engraftment was documented only in four patients with subsequent rejection, whereas the remaining five patients never attained sustained haemopoiesis. Rejection occurred in 6/67 (9%) patients transplanted with Campath 1M treated marrow: 2/54 (4%) with Campath 1G treated marrow and 1/10 patients transplanted with marrow treated with E-rosettes. Seven had ANLL and three were in advanced stage. The median age was 30 and only two patients were below age 24. An attempted second allograft failed in eight and was successful in only one patient who later died of interstitial pneumonitis.

CMi by T-cell repletion using donor’s PBL
Of 67 patients transplanted with Campath 1M treated marrow, 38 received early CMi with donor’s PBL (Table III). 16 patients were able to complete the scheduled T-cell repletion, but in 12 patients CMi was discontinued due to GVHD. Of the 54 patients transplanted with Campath 1G treated marrow scheduled to receive donor T cells, 11 were excluded: nine because of acute GVHD which appeared before day +28 and therefore they were not eligible for further therapy, and two others due to donor’s intercurrent infection. In 16/43 patients treated CMi was discontinued due to GVHD.

Acute GVHD
Overall, 50 recipients of Campath 1 treated marrow (41-3%)

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Table II. Methods of T-cell depletion and rate of graft failure among 131 allograft recipients with acute leukaemia.

<table>
<thead>
<tr>
<th></th>
<th>Campath 1M</th>
<th>Campath 1G</th>
<th>E-rosettes + SBA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>67</td>
<td>54</td>
<td>10</td>
</tr>
<tr>
<td>ANLL</td>
<td>45</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>First CR</td>
<td>32</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Second CR</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Advanced</td>
<td>9</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>ALL</td>
<td>22</td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>First CR</td>
<td>6</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Second CR</td>
<td>10</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Advanced</td>
<td>6</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Cells infused mean x 10^8 kg</td>
<td>2.16 ± 1</td>
<td>4.1 ± 1.7</td>
<td>0.9 ± 1.1</td>
</tr>
<tr>
<td>Rejection</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

* Campath 1M (lgM) was added into marrow buffy coat fraction. In vitro lysis took place in the presence of fresh donor serum as a source of complement.

† Campath 1G (lgG2b) was added into the marrow bag and infused without further manipulation.
Table III. Post-transplant repletion of donor T cells and its effect on remission and GVHD.

<table>
<thead>
<tr>
<th></th>
<th>Campath 1M (n = 67)</th>
<th>( p^2 )</th>
<th>Campath 1G (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-cell repletion (TCR)</td>
<td>Yes</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>No. of patients</td>
<td>38</td>
<td>29</td>
<td>43</td>
</tr>
<tr>
<td>Remission probability*</td>
<td>0.78</td>
<td>0.60</td>
<td>0.20*</td>
</tr>
<tr>
<td>Completed TCR</td>
<td>16</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>Discontinued TCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Due to acute GVHD</td>
<td>12</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Other causes$</td>
<td>10</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>AGVHD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>0</td>
<td>&lt;0.0001(\¶)</td>
</tr>
<tr>
<td>No</td>
<td>22</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>&gt;Moderate/severe</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CCVHD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>No</td>
<td>34</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>&gt;Moderate/severe</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

\* Seven patients were excluded from late TCR, in nine because GVHD developed before day +28.
\* Probability of remaining in remission at 2 years.
\¶ \( p \) value determined by Mantel-Haenszel test.
\$ Rejection, four patients; relapse, three patients; early death, four patients; minor donor, one patient.
\¶ \( p \) value determined by Fisher test.

Developed acute GVHD, most of them after receiving donor’s PBL. Patients receiving donor’s T cells had significantly higher risk to develop GVHD than patients not receiving donor’s T cells \((p^2 < 0.0001)\), irrespective of the type of antibody used. The incidence of GVHD was significantly higher in patients transplanted with Campath 1G treated marrow (63%) than with Campath 1M treated marrow (24%, \( p^2 < 0.0001 \)).

The probability of developing acute GVHD was similar in patients conditioned with TBI or BU-CY only, but among those receiving TBI, administration of more aggressive chemotherapy was associated with higher incidence of GVHD \((p < 0.001)\). There was no difference in the overall incidence of acute GVHD among the two types of leukaemia irrespective of the stage of disease, and no statistically significant differences between the various age groups with respect to the risk of GVHD.

Among 67 patients transplanted with marrow purged with Campath 1M (Table III), no acute GVHD was observed unless donor’s T lymphocytes were administered \((p^2 = 0.0001)\). Among those receiving donor cells, 16 patients developed acute GVHD of which three were severe. Among Campath 1G treated allograft recipients, nine patients whose marrows were treated with low concentration of antibody developed acute GVHD before any T cells were administered. Among the remaining 43 patients who received donor lymphocytes, acute GVHD occurred in 23.

**Chronic GVHD**

Chronic GVHD occurred in 28 recipients of Campath 1 treated marrows, 21 of whom received donor’s PBL (Table III). In 10 patients chronic GVHD represented the initial manifestation of the disease, whereas in others it was a progression of acute GVHD. The incidence of chronic GVHD among patients treated with donor’s PBL was higher in recipients of Campath 1G when compared with Campath 1M treated marrow.

There was no difference in the overall incidence of chronic GVHD among patients with ALL and ANLL and no difference between the various age groups. The incidence of chronic GVHD was similar in patients conditioned with TBI containing regimen and BU-CY only, and in the case of acute GVHD, chronic GVHD occurred predominantly in patients receiving TBI with triple chemotherapy as compared with patients receiving single or double chemotherapy \((p^2 = 0.0002)\).

**Event-free survival**

The overall event-free survival at 2 years of patients transplanted in first CR was 48%. In second CR 40% \((p^2 = 0.77)\) for first versus second CR), and in advanced disease 24% \((p^2 = 0.032\) for second CR versus advanced stage). There were no differences in event-free survival of patients transplanted in first or second remission in either type of leukaemia, but in both ALL and ANLL patients in advanced stage had significantly worse survival when compared with the two earlier stages combined: 19% vs 45%, respectively at 2 years in ALL \((p^2 = 0.018)\) and 30% vs 50%, respectively at 2 years in ANLL \((p^2 = 0.05)\). Among the variables analysed, age was the only other factor associated with improved survival: patients below 20 years of age had significantly better outcome compared to older patients \((p^2 = 0.038)\). Conditioning regimen, type of antibody, donor T-cells repletion and occurrence of GVHD had no effect on the overall event-free survival.
Cox proportional hazards model analysis demonstrated that factors associated with significantly worse favourable outcome were advanced stage of disease with relative risk of 2·68 compared to earlier stages \( (P^2 = 0·004) \), and age older than 10 years compared to younger patients (relative risk = 1·86, \( P^2 = 0·037 \)). Patients who developed chronic GVHD showed reduced risk of developing a relapse or dying compared with those who did not (relative risk = 0·54, \( P^2 = 0·0009 \)).

The probability of remaining in remission
The probability of remaining in remission by 2 years for patients receiving TCD allografts was 83% and 76% for first and second CR respectively \( (P^2 = 0·34) \) and 42% in advanced stage \( (P^2 = 0·009) \) (Fig 1). For ANLL patients transplanted in first or second CR the probability was 86% by 2 years compared with 51% for patients in advance stage \( (P^2 = 0·0012) \) (Fig 2). ALL patients transplanted in first or second remission had similarly higher 2-year probability of remaining in remission (72%) than patients transplanted in advanced stage (33%, \( P^2 = 0·0036 \)) (Fig 3).

For the 121 recipients of CamPATH 1 treated marrow the probability of remission was similar: for ANLL patients in first and second remission 84% by 2 years compared with
52% in advanced disease ($P^2 = 0.0065$), and for all patients 80% by 2 years for first and second remission and 29% for advanced disease ($P^2 = 0.001$).

The effect of donor’s T cell administration. The administration of donor T cells to 81 patients receiving T-cell-depleted marrow allografts using any Campath antibody had no significant benefits on their overall probability of relapse compared to 40 patients transplanted with T-cell-depleted marrow without T-cell repletion. The 2-year probability of relapse in patients treated with donor T cells was 25% compared with 32% without T-cell repletion ($P^2 = 0.64$). Among recipients of Campath 1M treated marrow, the probability of remaining in remission was higher among those receiving donor T lymphocytes (78% at 2 years) than without addition of donor T cells (60% at 2 years), although this difference was not significant ($P^2 = 0.20$, Fig 4).

Patients in first CR treated with donor T cells were more likely to remain in remission when compared to those untreated (90% vs 74% at 2 years respectively), regardless of whether or not they develop GVHD, but this difference is not significant. For the stage of leukaemia, all patients who received donor T cells and developed GVHD had only a 14% chance of relapse by 2 years, compared with 61% for patients who received T cells but had no GVHD ($P^2 = 0.013$), and 56% for patients who had neither T cells nor GVHD ($P^2 = 0.021$). No such difference was noticed in patients with ANLL.

The effect of GVHD. Patients in all disease categories had higher probability of remaining in remission if they developed any form of GVHD (84% at 2 years) compared with patients without any sign of GVHD (63%, $P^2 = 0.046$). Acute GVHD did not increase the probability of remaining in remission in any stage category; however, patients with ALL who developed GVHD seemed to have improved leukaemia-free survival than those without ($P^2 = 0.08$). Chronic GVHD had a general beneficial effect on the probability of remaining in remission ($P^2 = 0.068$), but it was significant only when patients were transplanted in first and second remission ($P^2 = 0.04$).

A multivariate Cox proportional hazards analysis was performed to determine factors associated with improved relapse-free survival. The risk factors studied included age, stage of leukaemia, conditioning protocol, type of T-cell depletion, CMV by donor’s PBL and GVHD. Age, type of antibody used for TCD, conditioning regimen, T-cell repletion by donor’s PBL and acute GVHD had no effect on leukaemia relapse-free survival. Stage of disease was found to be a significant factor with relative risk of 5.3 for developing relapse with more advanced stage compared with less advanced stage, along with chronic GVHD which had a relative low risk of 0.52 of developing leukaemic relapse.

Transplant-related mortality
Eighty-two patients died, 24 (29%) as result of leukaemic relapse. Six patients treated with donor T cells died of GVHD. Another 14 died of GVHD-associated CMV infection and liver failure, nine of whom were treated with donor T cells. Nine patients died as a result of graft failure, four treated with donor’s PBL. 15 other patients died as a result of organ failure attributed to the toxicity of the conditioning therapy, and nine from CMV and IP, irrespective of type of Campath used or donor T cell administration. In five other patients the cause of death was not determined.

DISCUSSION
The present report summarizes our results in a non-homogenous cohort of patients with acute leukaemia receiving T-cell-depleted allogeneic bone marrow transplant using Campath 1 antibodies and CMF using donors’ PBL to induce GVHD effect. Our preliminary data suggest that donors’ T lymphocytes mediate their antileukaemic activity in parallel with clinically evident GVHD, and that this effect is observed predominantly in ALL.

We have previously shown that Campath 1M is a safe, technically simple, and effective method for in vitro removal of lymphocytes from the bone marrow prior to transplantation. Campath 1M and more recently Campath 1G, have been widely used now in over 15 transplant centres (Hale et al, 1988; Hale & Wakhmann, 1988). We report here 121 leukaemia patients who received Campath 1 treated marrow, which also comprises the largest single-centre study using this antibody.

Campath 1 antibodies recognize CDw52, expressed abundantly on T and B cells and weakly on macrophages and NK cells (Hale et al, 1983; Cobbold et al, 1986). The IgM isotype is a potent lytic antibody which, in the presence of donor’s serum as a source of complement, effectively lyses T
cells in vitro. The major advantage of the IgG2b isotype, introduced in 1987, is the simplicity of using it. We have developed a simple 'Campath 1G in the bag' method (Naparstek et al., 1989), which requires only adding the antibody to the marrow bag without further in vitro manipulations. This antibody, when infused into the recipient with the marrow, has the capacity to kill both donor and host lymphocytes, most likely by antibody-dependent-cell-mediated cytotoxicity, thereby preventing GVHD as well as graft rejection by residual host lymphocytes (Slavin et al., 1986; Cobb et al., 1986; Hale et al., 1988). The use of Campath 1M historically preceded 1G, therefore the patients were not randomized, and no comparison of these two antibodies is possible. Nevertheless, regardless of the isotype used, the survival and the incidence of leukaemia relapse were similar.

Eight recipients of Campath 1 treated marrows (6-6%) rejected their graft: six received their marrow treated with Campath 1M and two with Campath 1G. This rejection rate is significantly less than in previously published reports on TCD BMT (Kerman et al., 1989; Bordignon et al., 1989; Patterson et al., 1986). Because residual immunocompetent host cells are believed to be responsible for graft rejection, the escalation of immunosuppression by addition of TIL to our conditioning regimen has resulted in improved rates of sustained engraftment (Slavin et al., 1984, 1986). This approach has been successfully employed by us in aplastic anaemia and also in genetic diseases (Slavin et al., 1982; Or et al., 1986).

Patients transplanted in advanced stage of disease show relatively high probability of remaining leukaemia free at 2 years: 42% (P = 0.009 compared to earlier stages). When analysed separately for AML and ALL, the probability of remaining in remission was 86% and 72% respectively for transplants conducted in first and second remission, and 51% and 33% in patients transplanted in advanced leukaemia. This improved relapse-free survival in high-risk patients may be attributed in part to post-transplant immunotherapy with donor T lymphocytes, more aggressive pretransplant antileukaemia conditioning, or a combination of these. Multivariate Cox proportional hazards analysis revealed that among the many factors analysed, stage of leukaemia and chronic GVHD were the only factors significantly associated with improved relapse-free survival.

Prevention of leukaemic relapse is a major aim in allogeneic BMT. It may be achieved either by intensifying the pretransplant chemoradiotherapy which has been shown to augment organ toxicity and predispose to early transplant-related death, or by intensifying the immunemediated interaction of donor's immunocompetent lymphocytes against residual tumour cells, i.e. the GVL effect. Previous attempts to use donor's buffy coat in recipients of non-TCD marrow allografts in patients treated with conventional GVHD prophylaxis did not result in any appreciable benefits (Sullivan et al., 1989c, Verdonck et al., 1990), most likely due to procedure-related toxicity, particularly GVHD in a significant proportion of patients (82% and 60% respectively).

Over the past few years cumulative clinical data from the International Bone Marrow Transplant Registry suggest that GVL can occur independently of GVHD (Horowitz et al., 1990). Studies in animal models also suggest that under certain experimental conditions GVL may be induced with no overt GVHD (Trott et al., 1987; Weiss et al., 1990; Kornfeld & Sprent, 1987; Slavin et al., 1990). Our results in experimental B-cell leukaemia/lymphoma in BALB/c mice have shown that GVL may be accomplished without GVHD in tolerant GVHD-free chimeras by delayed post-transplant administration of donor-type lymphocytes, thus establishing our rationale for post-transplant CMI (Slavin et al., 1981, 1992; Weiss et al., 1992).

In our non-prospectively randomized heterogeneous group of patients, only recipients of Campath 1M treated marrow were partially informative for the role of cell-mediated immunotherapy using donor's PBL for prevention of relapse. Of 67 patients, only 38 were scheduled to receive CML, therefore the remaining 29 patients serve as historical controls. Due to the strong lytic activity of Campath 1M in vitro, resulting in 3–4 log depletion of T cells, the incidence of GVHD was extremely low in this group (Table III). Post-transplant administration of donor's PBL resulted in clinically evident GVHD in 42%, but was associated with higher probability of remaining in remission as noted predominantly in high-risk patients and in patients with ALL. Therefore, under the experimental conditions investigated, donor T lymphocytes seem to modulate antileukaemic activity when accompanied by clinically evident GVHD. GVHD could be controlled in most patients by withholding subsequent doses of immunocompetent lymphocytes. However, patient variability unfortunately does not permit accurate prediction of the individual threshold of the cell dose that leads to GVH or GVL effect, and indeed, GVHD was fatal in three patients. Effective GVL independent of GVHD was therefore not demonstrated.

The interpretation of the higher occurrence of GVHD in patients transplanted with Campath 1G treated marrow is less conclusive. The T-lymphocyte depletion using 1G antibody 'in the bag', depends largely upon the efficacy of the in vivo effector mechanisms (ADCC), the activity of the reticuloendothelial system of the recipient, and antibody concentration. Because we noticed that low antibody concentration (0.3 mg/10^6 cells) is insufficient to completely prevent GVHD, early T-cell repletion in this group of patients may have accelerated ongoing subclinical donor-versus-host responses, and GVHD was indeed observed in a substantial proportion. Smaller doses of T cells and/or delayed TCR might have resulted in more effective CMI without severe GVHD. Further prospective studies using different concentrations of Campath 1G 'in the bag' are required to confirm the optimal conditions for prevention of GVHD using this simplified method for TCD.

The onset and the intensity of GVHD in TCD allograft recipients seem related to the timing as well as to the proportion of T cells administered in relation to BMT (Slavin et al., 1978). We intend to improve the CMI by optimizing the schedule of administration of donor's T cells to be given, in an attempt to further intensify the graft-versus-
leukemia effect without augmenting GVHD. In future, CMI could be further improved by administration of well-defined lymphocyte subsets or possibly by in vitro and/or in vivo activation of antileukemia effector cells of T and possibly NK lineages.

The encouraging preliminary results, and the recent observations suggesting that donor's PBL are useful in eradication of leukemic cells in patients relapsing after allogeneic BMT, suggest that cell therapy may represent a novel and effective approach not only for treatment but for prevention of relapse in malignant haematological diseases.

REFERENCES


Meeting report

Recent results using CAMPATH-1 antibodies to control GVHD and graft rejection

G Hale and H Waldmann for CAMPATH users

Department of Pathology, University of Cambridge; Dunn School of Pathology, University of Oxford, UK

Summary:

Recent data from the CAMPATH users group are reported. Different protocols have been tested using CD52 antibodies CAMPATH-1M and CAMPATH-1G for prevention of GVHD and graft rejection in allogeneic transplants from both sibling and volunteer unrelated donors. Leukaemia relapse remains a significant problem for patients with CML, but in other diseases the recent results using T cell depletion appear to be as good as, or better than, published data with conventional GVHD prophylaxis. In addition, the morbidity and mortality associated with chronic GVHD are substantially reduced. Future collaborative studies to consolidate these findings include a randomised trial of the humanised antibody CAMPATH-1H organised under the auspices of the EBMT. There are also plans to carry out experimental studies using CAMPATH-1 antibodies to deplete T cells from peripheral blood stem cell harvests.

Keywords: CAMPATH; GVHD; graft rejection

The merits and drawbacks of T cell depletion in allogeneic marrow transplantation have been debated for the last 10 years without consensus. It is clear that removal of donor T cells, by whatever means, is the most effective way of preventing graft-versus-host disease, but there is a potential penalty of graft rejection and loss of a beneficial graft-versus-leukaemia effect. The largest studies have been carried out using CAMPATH-1 (CD52) monoclonal antibodies for T cell depletion and a co-operative group consisting of 40-50 bone marrow transplant centres has been working and meeting together since 1986 to evaluate different ways of using CAMPATH-1 antibodies to the best advantage. On 7 January 1995 the CAMPATH users group met in Cambridge, UK to review the recent results. To date CAMPATH-1 antibodies have been used in over 2000 bone marrow transplants for the prevention of graft-versus-host disease and graft rejection. The clinical data vindicate the concept that these complications can most effectively be prevented by depletion of T lymphocytes from both donor and recipient. However, they also show that donor lymphocytes can, under some circumstances, contribute an anti-leukaemia effect and that lymphocyte depletion can lead to subtle changes in the speed of engraftment and of viral reactivation. Thus there is a fine balance between GVH, HVG, relapse and infection. Various protocols of lymphocyte depletion have been tested, using either CAMPATH-1M (IgM) plus complement or CAMPATH-1G (IgG2b) to treat the donor bone marrow ex vivo and CAMPATH-1G in vivo to treat the recipient (Figure 1, Table 1).

For transplants from HLA-identical siblings, the best results reported by the CAMPATH users have been obtained with either of two protocols:

02 CAMPATH-1M plus complement for T cell depletion combined with CAMPATH-1G in vivo (day -10 to day -6) to prevent graft rejection (Royal Free Hospital, London, UK, University Hospital, Ulm, Germany and King Faisal Hospital, Riyadh, Saudi Arabia).

03 CAMPATH-1G in the bag to deplete donor T cells and possibly contribute to host immunosuppression. Pre-transplant conditioning also included total nodal irradiation in order to reduce the risk of graft rejection (Hadassah University Hospital, Jerusalem, Israel and University Hospital, Cape Town, South Africa).

In both, the transplant complications have been very low (less than 10% combined risk of graft failure or severe grade 3-4 GVHD) and leukaemia-free survival has been good (approximately 60% at 2 years for patients with acute leukaemia transplanted in first remission). These results are significantly better than with depletion of donor T cells alone (Figure 2) and compare favourably with published data using CsA/MTX for GVHD prophylaxis since chronic GVHD was virtually eliminated.

Other teams (University Hospital, Leiden, The Netherlands and Addenbrookes Hospital, Cambridge, UK) have tested the combination of CAMPATH-1G in the bag for T cell depletion plus CAMPATH-1G in vivo (instead of TLI) to prevent rejection (Protocol 07). Unexpectedly, this resulted in significantly delayed engraftment and a high incidence (approximately 20%) of graft failure. Since CAMPATH-1 antibodies did not appear to be directly toxic to stem cells in any of the other clinical studies, this result was hard to explain. One hypothesis previously suggested was that
Figure 1. Protocols of antibody treatment for prevention of GVHD and graft rejection. The approximate timing of in vivo antibody treatment with Campath-1G is shown. The dose was usually 5-10 mg/day.

Table 1. Graft failure and GVHD according to antibody protocol for HLA-matched sibling transplants for malignant diseases (no post-transplant immunosuppression)

<table>
<thead>
<tr>
<th>No.</th>
<th>In vivo</th>
<th>In vitro</th>
<th>No of patients</th>
<th>Graft failure (P)</th>
<th>Percentages</th>
<th>Actuarial at 2 years ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute GVHD</td>
<td>Chronic GVHD</td>
<td>Either (P)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/3/4 (P)</td>
<td>M/S (P)</td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>None</td>
<td>CP1M</td>
<td>290</td>
<td>12</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>02</td>
<td>CP1G</td>
<td>CP1M</td>
<td>161</td>
<td>8 (0.001)</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>03</td>
<td>None</td>
<td>CP1G</td>
<td>53</td>
<td>4 (0.004)</td>
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<td>7</td>
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<td>CP1G</td>
<td>46</td>
<td>22</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are compared with the first group (Protocol 01). Those which are significantly better are highlighted in bold script, those which are worse are in italic script. In each case the P value is also given. Although the data presented in this table are of only patients who received no post-transplant immunosuppression, essentially identical results were obtained when all patients were analysed irrespective of post-transplant immunosuppression. M = moderate; S = severe.
Figure 2 Leukaemia-free survival for patients with malignant disease transplanted from HLA-matched siblings. (a) Comparison of Protocol 02 and Protocol 01; (b) comparison of Protocol 03 and Protocol 01.

there may be a low, but important contribution from lymphocyte activation (either donor or recipient) towards stimulation of haemopoiesis during the immediate post-transplant period.1

Some complications of antibody therapy were not seen uniformly at all centres. For example, delayed engraftment was observed by most groups using Campath-1G in vivo for treating matched siblings, but this was not the case in many of the transplants from unrelated donors. One centre (Ulm) reported an increase in the frequency and speed of CMV reactivation following therapy with Campath-1G. In most patients the infection was controlled, possibly as a result of activation of donor NK cells. These cells may also have contributed to the low rate of relapse reported at that centre. However, this reactivation of CMV did not seem to be a universal phenomenon since other groups found it to be quite rare. An understanding of the reasons for these differences might give us useful insights into the biology.

In patients transplanted for CML from matched siblings, T cell depletion results in a substantially increased risk of relapse and therefore it is now rarely used in this setting. In contrast, there is only a modest increase in relapse risk for patients with acute leukaemia.1,10 Some teams (notably Hadassah Hospital, Jerusalem) have tried to induce a beneficial graft-versus-leukaemia effect by graded infusion of donor lymphocytes following the transplant11,12 and others have added back small numbers of lymphocytes or unfractionated marrow after T cell depletion. Combined analysis of all these studies shows that the addition of donor T cells does give a small, but significant reduction in the risk of relapse (from about 38% to 26% at 2 years) when data for all patients transplanted for acute leukaemia are pooled. However, the addition of T cells is also associated with an increase in acute and chronic GVHD and an increase in transplant-related mortality so the overall leukaemia-free survival is virtually identical. Although this approach does not yet seem to give an overall benefit, the hope is that it may be more tolerable and effective to give donor T cells once the graft is well established.

In recent years a major application of Campath-1 antibodies has been in treating patients transplanted from volunteer unrelated donors.2,13 Children (mostly with ALL in CR2 or more advanced) have largely been treated with Campath-1G in vivo plus Campath-1M ex vivo (Protocol 02) and this has proved to be quite successful (Royal Hospital for Sick Children, Bristol, UK, Royal Free Hospital, London, UK). In Bristol, the incidence of graft failure was 12%, of severe GVHD 13%, and of relapse (at 2 years) 23%. Survival (at 2 years) was approximately 48%. So far there are few published data using conventional GVHD prophylaxis with which to compare the results but they compare favourably with transplants from HLA-matched siblings.

Adults receiving VUD transplants have mostly been treated with just Campath-1G in vivo both before and after the transplant (Protocol 06). The donor marrow has not been manipulated but conventional GVHD prophylaxis of CSA and MTX has been given. This protocol, rather than the more radical one was adopted for two reasons. First, the preliminary results indicated a trend towards better leukaemia-free survival, and second the majority of the patients suffered from CML and it was feared that T cell depletion would incur an unacceptable risk of relapse. For patients transplanted for CML in first chronic phase the incidence of graft failure was 16%, of severe GVHD 25%, and of relapse (at 2 years) 23%. Leukaemia-free survival (at 2 years) was 46%, which is similar to that reported for VUD transplants using conventional GVHD prophylaxis.14 As expected, the incidence of severe GVHD is lower and relapse is higher following the in vivo T cell depletion. The net impact on morbidity and mortality in the longer term is hard to gauge. A randomised trial of this protocol compared with standard treatment has been initiated by the Hammersmith team for the Chronic Leukaemia Working Party of the EBMT. This study will use the humoral antibody Campath-1H, for which there is a prospect of commercial production in the long run. All centres who are members of the EBMT are invited to enrol patients in this study.

Currently there is a great deal of interest in transplantation using peripheral blood stem cells. It has already been shown that Campath-1H is effective for depletion of T cells15 in this setting and studies are now planned to test the IgG antibodies, particularly Campath-1H for this application. It seems reasonable to suppose that in vitro depletion would be the best way of preventing GVHD after allogeneic transplantation of PBSC and this approach may eventually be preferable to the use of bone marrow.

Graft-versus-host disease is still a potentially horrendous
complication of BMT and, particularly in its chronic form, leads to severe morbidity and a poor quality of life for many patients. This is what motivates so many teams to persevere with T cell depletion, despite the potential disadvantages. With new developments in the techniques for monitoring residual disease it is hoped that patients might be spared the major risks associated with immune reactions during the immediate post-transplant period, and perhaps be treated with more selective immunotherapy using donor leukocytes only in the event of impending clinical relapse.

Acknowledgements

We are grateful to the staff of the TAC, Jenny Phillips, Annamarie Drumm, Patrick Harrison, Donna Stock, Angela Shaw and Jeremy Hoigate, for their work producing Campath-1 antibodies.

We also gratefully acknowledge the efforts of the many members of the following bone marrow transplant teams who contributed to the clinical care, analytical measurements and data collection: Birmingham Children’s Hospital, Birmingham, UK; Queen Elizabeth Medical Centre, Birmingham, UK; Institute of Haematology Seragolli, Bologna, Italy; Royal Bournemouth Hospital, Bournemouth, UK; Royal Hospital for Sick Children, Bristol, UK; Jules Bordet Hospital, Brussels, Belgium; Addenbrooke's Hospital, Cambridge, UK; University of Cape Town Leukaemia Centre and Department of Haematology, Groote Schuur Hospital, Cape Town, South Africa; University of Wales Medical School, Cardiff, UK; Royal Devon and Exeter Hospital (Wonford), Exeter, UK; Hospital Cantonal, Geneva, Switzerland; San Martino Hospital, Genoa, Italy; Department of Paediatrics, University of Gothenburg, Gothenburg, Sweden; Hadassah University Hospital, Jerusalem, Israel; Royal Free Hospital, London, UK; St George’s Hospital, London, UK; Hammersmith Hospital, London, UK; Institute of Child Health, London, UK; The London Clinic, London, UK; Royal Marsden Hospital, London, UK; University College Hospital, London, UK; Westminster Hospital, London, UK; St. James University Hospital, Leeds, UK; Royal Infirmary, Leicester, UK; Departments of Haematology and Paediatrics, University Hospital, Leiden, The Netherlands; Royal Infirmary, Liverpool, UK; Mainz Children’s Hospital, Mainz, Germany; Royal Manchester Children’s Hospital, Manchester, UK; Christie Hospital, Manchester, UK; Manchester Royal Infirmary, Manchester, UK; Ludwig-Maximilians University Hospital, Munich, Germany; Newcastle General Hospital, Newcastle, UK; City Hospital, Nottingham, UK; John Radcliffe Hospital, Oxford, UK; Hospital Necker, Paris, France; Policlinico S Matteo, Pavia, Italy; Royal Perth Hospital, Perth, Australia; King Faisal Hospital, Riyadh, Saudi Arabia; University degli Studi La Sapienza, Rome, Italy; Medico Marques de Valdecilla, Santander, Spain; St Vincent’s Hospital, Sydney, Australia; University of Ulm, Ulm, Germany.

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References

Campath-1G in vivo confers a low incidence of graft-versus-host disease associated with a high incidence of mixed chimaerism after bone marrow transplantation for severe aplastic anaemia using HLA-identical sibling donors.


Department of Haematology and Child Health, St George's Hospital, London, UK.

We have evaluated the effect of in vivo Campath-1G on engraftment and GVHD in 23 patients with severe aplastic anaemia transplanted from HLA-identical sibling donors. In 14 patients Campath 1G was given pre-transplant for up to 9 days in an attempt to overcome graft rejection (group 1). In nine patients Campath-1G was given pre-transplant, but also continued post-transplant until day +5 to reduce GVHD (group 2). There were three patients with late graft failure in group I following initial neutrophil engraftment, and four cases of grade II+ GVHD. In group II, two patients had early graft failure (no take), and there were no cases of acute GVHD out of seven evaluable patients. One patient in group I developed chronic GVHD of the liver, and two patients (one in each group) had transient localised chronic GVHD. PCR of short tandem repeats was used to evaluate chimaeric status in 13 patients. Of 11 patients with initial neutrophil engraftment, only one had 100% donor haemopoiesis at all times. The remaining patients had either transient mixed chimaerism or persistence of recipient (< 20%) cells. We conclude that in vivo Campath-1G is associated with a high incidence of mixed chimaerism which tips the balance away from GVHD but towards graft rejection.

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T cell depletion

Excessive T cell depletion of peripheral blood stem cells has an adverse effect upon outcome following allogeneic stem cell transplantation

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¹Department of Haematology, University College London, London, UK; and ²Sir William Dunn School of Pathology, University of Oxford, UK

Summary:

We evaluated the outcome of two modes of T cell depletion for HLA-identical sibling stem cell transplants in 34 consecutive adult patients: group A (n = 11) received PBSCT post ClinIMACS immuno-magnetic enrichment of CD34⁺ cells and group B (n = 23) received bone marrow following in vitro incubation with CAMPATH-1M and complement. All patients received an identical conditioning regimen which consisted of in vivo CAMPATH-1H 20 mg over 5 days, thiopeta 10 mg/kg, cyclophosphamide 120 mg/kg and 14.4 Gy TBI. No additional graft-versus-host disease prophylaxis was given. The mean T cell dose administered was 0.02 ± 0.05 x 10⁹/kg for group A and 2.8 ± 2.8 x 10⁹/kg for group B (P < 0.001). With a median follow-up of 28 months overall survival was 36.4% for group A at 12 months compared to 73.3% for group B (P = 0.001). Transplant-related mortality in group A at 12 months was 63.6% as compared to 18.0% in group B (P = 0.003). Most of the procedure-related deaths in group A occurred secondary to infection. These results suggest that extensive in vitro T cell depletion of peripheral blood stem cells in combination with in vivo T cell depletion may have profound effects upon the incidence of infections following allogeneic stem cell transplantation and this may adversely affect transplant-related mortality. Bone Marrow Transplantation (2001) 28, 827–834.

Keywords: CD34 selection; allogeneic transplantation; T cell depletion

T cell depletion (TCD) is an effective means of reducing the incidence and severity of acute or chronic graft-versus-host disease (GVHD) following allogeneic stem cell transplantation (SCT).¹,² A number of different methods for TCD have been developed over the last two decades that include counter-flow elutriation,³ lectin agglutination,⁴ monoclonal antibodies directed at T cell antigens with narrow⁵ or broad specificity⁶ or depletion through positive selection of CD34⁺ stem cells.⁷ The clinical benefit of TCD allogeneic SCT is controversial since this approach may be compromised by increased rates of graft rejection⁸ and a higher rate of relapse.⁹ To overcome the risk of graft rejection additional approaches have been adopted that include increasing pre-transplant immunosuppression,¹⁰ 'partial' TCD¹¹ or increasing the number of stem cells administered by using peripheral blood stem cells (PBSCs).¹²,¹³ The optimal T cell content of a graft that maintains a significant graft-versus-leukaemia (GVL) effect has not yet been defined. Although TCD is used widely by many different transplant centres, few studies have assessed directly how different modes of TCD affect clinical outcome.

One area of potential concern is the significant delay in immune reconstitution that occurs following TCD allogeneic SCT. This is particularly the case in adults, where the initial T cell repertoire is dependent upon peripheral expansion of mature T cells in the graft.¹⁴–¹⁶ Delayed recovery of CD4⁺, CD4⁺CD45RA⁺ and TCRγδ T cells with a concomitant reduction in TCR diversity are typical features of the early post-transplant period following TCD allogeneic SCT.¹⁶–¹⁸ T cell purging may thus exacerbate post-transplant immunodeficiency and be complicated by an increased incidence of opportunistic infections, particularly CMV re-activation.²⁰

In this report, we highlight the major impact on clinical outcome of two approaches to in vitro TCD, using either positive selection of CD34⁺ cells from PBSCT or CAMPATH 1M treatment of bone marrow, as part of a protocol that employed additional in vivo TCD. Patients who received donor PBSCT heavily depleted of T cells (5 log) by immuno-magnetic selection of CD34⁺ cells had a substantially higher procedure-related mortality than recipients of CAMPATH 1M-treated bone marrow. The higher number of procedure-related deaths was caused by a marked increase in the number of opportunistic infections within the TCD PBSCT group. This study suggests that following HLA-identical sibling SCT in adults, extensive in vitro TCD of PBSCT in combination with in vivo TCD is compli-
cuted by a profound immunodeficiency that outweighs any benefit in terms of reduction of GVHD.

Patients and methods

A total of 34 consecutive adult patients who received a TCD HLA-identical sibling donor stem cell transplant at University College Hospital London, UK between January 1997 and October 1999, were included in the analysis. During this period, the method of in vitro TCD was changed due to an insufficient supply of CAMPATH 1M. The protocols were approved by the local institutional review committees and all patients gave informed consent. Two groups of patients were defined according to the mode of T cell depletion employed: ‘group A’ consisting of 11 patients who received PBSC following ClinIMACS immuno-magnetic enrichment of CD34+ cells and ‘group B’ comprising 23 who received bone marrow following in vitro incubation with CAMPATH 1M and complement.

Patient characteristics

The patient characteristics for each group are shown in Table 1. Patients were considered to be ‘standard risk’ in the case of acute myeloid leukaemia (AML) in complete remission (CR) or chronic myeloid leukaemia (CML) in first chronic phase. All other diagnoses were considered high risk. There were no significant differences between the groups in terms of sex, performance status at the time of transplant, time to transplant or those at risk of CMV disease (Table 1). However, only one patient in group A was considered to be high risk as compared to 11 of 23 patients in group B (P = 0.05). This reflected, in part, the lack of patients with lymphoproliferative disorders in group A. Three patients in group B had received a previous SCT and none in group A.

Antibodies

CAMPATH-1H is a humanized IgG1 monoclonal antibody against the CD52 antigen. It was prepared from the culture supernatant of Chinese hamster ovary cell transfectants cultured in a hollow fibre fermenter. It was purified by affinity chromatography on Protein A sepharose and size exclusion chromatography on Superdex 200 and formulated in phosphate-buffered saline. The half-life of CAMPATH-1H in humans is dependent on the amount of target CD52 antigen in the patient. Based on work in progress, there is persistence of CAMPATH-1H in vivo past day 0 sufficient to cause T cell lysis by ADCC. CAMPATH-1M is a rat IgM antibody that recognises the same antigen. It was prepared from hybrid myeloma cells using stimulated ferments, purified by fractionation with ammonium sulphate and reconstituted in phosphate-buffered saline.

Conditioning regimen

All patients received the same conditioning regimen which consisted of in vivo CAMPATH-1H 20 mg on days 9 to 12, thiopenta 5 mg/kg on days 1 and 2, cyclophosphamide 60 mg/kg on days 5 and 6 and 14.4 Gy total body irradiation, with partial lung shielding, in eight fractions over 4 days.

For stem cell collection for group A patients, sibling donors received G-CSF at 10 μg/kg subcutaneously once daily on day 4 to day 0. Leukaphereses were performed on day 0 + 1 using conventional techniques for PBSC. TCD was performed by positive immuno-magnetic selection of CD34+ cells using a ClinIMACS, (Miltenyi Biotec, Bergisch Gladbach, Germany) cell separation system.

For group B patients, donor bone marrow was aspirated under general anaesthesia and TCD performed in vitro upon the derived buffy coats by incubation with 25 mg CAMPATH 1M at room temperature for 10 min followed by incubation with 10-30% autologous plasma at 37°C for 45 min.

For both protocols, the level of TCD was monitored by flow cytometric analysis of CD3 staining.

Supportive care

Patients were managed in reverse isolation in conventional or laminar air flow rooms. All patients received prophylaxis with cotrimoxazole or pentamidine against Pneumocystis carinii infection. Azithromycin and trimethoprim prophylaxis were routinely used. Blood products were irradiated to 25 Gy. Red cell and platelet transfusions were given to maintain the Hb >9 g/dl and platelet count >10 x 10^9/L. Patients who were CMV seronegative received only blood products from CMV seronegative donors; seropositive patients received blood products from donors unscreened for CMV. Fibricle neutropenic patients received intravenous piperazobactam and gentamicin as first line antibiotic therapy. Patients received G-CSF at 5 μg/kg/day from day 4+6 until

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of groups A and B</th>
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<tr>
<td></td>
<td>Group A (n = 11)</td>
</tr>
<tr>
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<tr>
<td>Median age years (range)</td>
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</tr>
<tr>
<td>Diagnosis (n)</td>
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</tr>
<tr>
<td>AML CR2</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>HD</td>
<td>0</td>
</tr>
<tr>
<td>HG-NHL</td>
<td>0</td>
</tr>
<tr>
<td>CMV 1st cp</td>
<td>2</td>
</tr>
<tr>
<td>CMV ap</td>
<td>0</td>
</tr>
<tr>
<td>Risk status standard/high</td>
<td>10/1</td>
</tr>
<tr>
<td>Median time to transplant, months (range)</td>
<td>6.5 (2-17)</td>
</tr>
<tr>
<td>Previous transplant</td>
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</tr>
<tr>
<td>CMV serology (n)</td>
<td></td>
</tr>
<tr>
<td>P-0</td>
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<tr>
<td>P-0+</td>
<td>4</td>
</tr>
<tr>
<td>P-0+</td>
<td>2</td>
</tr>
</tbody>
</table>

MDS = myelodysplastic syndrome; MM = multiple myeloma; HD = Hodgkin's disease; HG-NHL = non-Hodgkin's lymphoma; 1st cp = first chronic phase; ap = accelerated phase; P = patient; D = donor.
the ANC was at least $1.0 \times 10^9/l$ for 2 consecutive days. CMV seropositive patients were monitored weekly from transplantation until at least day 120 by qualitative PCR of CMV DNA from peripheral blood. Pre-emptive ganciclovir therapy (5 mg/kg twice daily intravenously or adjusted according to renal function) was given following two consecutive positive PCR results and discontinued after 2 weeks if a negative PCR was obtained.23 In the event of continued PCR positivity, fosarnet was substituted for ganciclovir and the drugs alternated every 2 weeks according to the PCR results. A single patient (UPN5) received cidofovir as initial pre-emptive therapy for PCR detection of CMV re-activation.

**GVHD prophylaxis and grading**

No additional GVHD prophylaxis was given. Patients who survived 100 days or longer were evaluated for chronic GVHD. Both acute GVHD and chronic GVHD were graded according to the consensus criteria.25-26

**Evaluation of infective complications**

An infectious complication was defined as any infection occurring post day 21 which required continued or new hospital admission/referral. CMV re-activation was defined as two consecutive peripheral blood PCR positive results. CMV disease was diagnosed on the basis of an inflammatory process due to CMV confirmed by the presence of typical cytopathic and immuno-fluorescent features in histological preparations or positive detection of early antigen fluorescent foci (DEAFF) and/or CMV culture from relevant material such as washings from broncho-alveolar lavage (BAL). Pulmonary fungal infection was diagnosed either by histological confirmation or characteristic high resolution CT appearances (halo sign) plus positive cultures from a BAL. Fungal infection at other sites was identified from post-mortem histological analysis of affected organs. RSV, parainfluenza I and II or influenza B infection were defined as pulmonary signs plus direct immuno-fluorescence and/or culture for the relevant viruses from nasso-pharyngeal aspirate or BAL. Confirmation of Legionnaire's disease was made by the presence of urinary Legionella antigen on two consecutive occasions and confirmed by post mortem histological examination of lung. Adenovirus, RSV and measles infection were confirmed by histological examination of the relevant organs postmortem. Pneumonia was defined as fever, associated with signs of lung consolidation and new infiltrates identified on chest X-ray or high resolution CT.

**Statistical analysis**

Overall survival (OS) was measured from transplantation until death from any cause. Patients still alive at the time of the analysis were censored at the last follow-up date. Transplant-related mortality (TRM) was determined from the date of transplantation until death related to transplantation. Patients who died from other causes were censored at the time of death. OS and TRM were estimated by the Kaplan-Meier method and the significance of differences between the curves was estimated by the log rank test. Patient characteristics in the two groups were compared by Fisher's exact test or the Mann-Whitney test, whichever was appropriate.

**Results**

**T cell depletion**

Patients in group A received approximately 2 log less T cells than group B patients (Table 2). The mean T cell dose was $0.02 \pm 0.05 \times 10^9/kg$ for group A recipients and $2.8 \pm 2.8 \times 10^9/kg$ for group B patients ($P < 0.001$). Three patients in group A had "add-back" of donor T cells following the transplant (Table 2). Two of these patients, UPN6 and UPN8, received $1 \times 10^9/kg$ T cells at 4 and 5 months.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age</th>
<th>Sex</th>
<th>Diagnosis</th>
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<th>T cell dose*</th>
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<tr>
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<td>51</td>
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*109 cell/kg recipient weight.

*Patients had donor lymphocyte infusions at following doses x 109 CD3 cells/kg recipient weight (time post transplant in months): UPN3 18; UPN5, 11; UPN6, 1; UPN7, 3; and UPN20, 22 (12+). MDS = myelodysplastic syndrome; RA = refractory anemia; RAEB = refractory anemia with excess blasts; MM = multiple myeloma; HD = Hodgkin's disease; HG-NHL = non-Hodgkin's lymphoma; 1st cp = first chronic phase; ap = accelerated phase; Rel = relapse; PR = partial response; ND = not done.
respectively, because of progressive pulmonary infections. UPN3 was given 3 × 10^9/kg T cells for cytogenetic relapse of CML at 10 months. Two patients in group B received donor T cells. UPN17 received 3 × 10^9/kg T cells at 11 months for relapsed AML and UPN20 received 2 × 10^9/kg T cells for treatment of relapsed multiple myeloma.

**Engraftment**

The mean CD34+ cell dose per patient was 4.4 ± 1.8 × 10^9/kg in group A and 1.7 ± 1.0 × 10^9/kg in group B (P < 0.001). Neutrophil engraftment occurred more rapidly in group A, with neutrophils >0.5 × 10^9 on day 12.0 ± 2.1 compared to day 17.2 ± 4.0 for group B (P < 0.001). One patient in group B failed to engraft and died on day +74. All patients in group A engrafted. No cases of secondary graft failure occurred in either group. No differences were observed between the groups in terms of transfusional independence by day 100 (four of seven evaluable patients in group A as compared to 15 of 22 evaluable patients in group B were transfusion independent).

**GVHD**

The incidence of acute GVHD was low in both groups (Table 3). No patients in group A developed acute GVHD of greater than grade 1 as compared to two of 23 patients in group B. For patients who survived day 100 no difference in either the incidence or extent of chronic GVHD was observed. Thus, three of seven evaluable patients in group A developed chronic GVHD (extensive in all cases) as compared to 11 of 22 patients in group B (extensive in three patients and limited in the remaining eight patients).

In both groups, patients who received T cell add-back post SCT were at high risk for the development of extensive chronic GVHD. Thus, four of five patients who received a donor T cell infusion following the transplant subsequently developed extensive chronic GVHD. One patient (UPN3) died of progressive GVHD at 12 months following SCT and 2 months following infusion of 3 × 10^9/kg donor T cells for relapsed CML. The remaining patients have all required prolonged treatment with prednisolone and cyclosporine.

**Infective complications**

Most of the deaths in group A occurred secondary to infection (Table 3). Thus, at 12 months six of 11 patients in group A died secondary to infection (CMV disease n = 2, invasive pulmonary aspergillosis (IPA) n = 1, RSV/measles pneumonia n = 1, Legionella n = 1, E. coli sepsis n = 1) compared to only one of 23 patients (adenovirus) in group B (P = 0.002).

The propensity to infection in group A is also highlighted by the greater number of documented serious infections post day 21 in group A than in group B (Table 4). Thus, with a median follow-up of 28 months, four of 11 patients in group A had three or more significant infections as compared to one of 23 in group B (P = 0.03). Furthermore, patients in group A were more likely to have co-existent infections, with six of 11 patients in group A having two or more simultaneous infections as compared to only two of 23 patients in group B (P = 0.005). In a number of patients from group A, infection progressed despite the appropriate anti-microbial therapy. Patients UPN2 and UPN6 both developed IPA which failed to respond to conventional, and then liposomal amphotericin therapy. Patient UPN2 also developed a large, deep skin ulcer secondary to HSV II (proven on culture and biopsy), which failed to respond to aciclovir, foscarnet or cidofovir. Patient UPN3 developed CMV colitis and pneumonitis despite preemptive therapy with foscarnet and cidofovir and subsequent therapy with ganciclovir and intravenous immunoglobulin.

There were no significant differences between the two groups in terms of the frequency of CMV reactivation in that five of six CMV seropositive individuals in group A and 11 of 19 in group B met the criteria for preemptive treatment for CMV reactivation or CMV disease. The median total duration of anti-CMV therapy administered was 98 days (range 8-127 days) in group A and 33 days in group B (range 13-129 days). Two patients from group A died from CMV disease in group A and none from group B.

**Immune reconstitution**

Group A patients showed a significant delay in the recovery of the absolute lymphocyte count following transplant. Thus, at 2 months post transplant the absolute lymphocyte count was >1.0 × 10^9/l in none of nine evaluable patients in group A but 10 of 21 patients in group B (P = 0.01). Absolute lymphocyte numbers for the first 5 months for both groups are shown in Figure 1. Analysis of T cell subsets at our centre is usually performed at 2 monthly intervals post transplant. The poor outcome of patients in group A meant that the majority were not evaluated and thus an evaluation of T cell subset reconstitution was not possible.

**Overall survival and transplant-related mortality**

With a median follow-up of 28 months the overall survival (OS) and TRM for all 36 patients at 12 months were 64.7% and 33.0%, respectively. However, there was a major difference in OS between the groups (Figure 2). Thus, at 12 months OS was 36.4% for group A and 78.8% for group B (P = 0.001). This difference was accounted for to a great extent by the high number of procedure-related deaths in group A (Figure 3). At 12 months the TRM was 63.6% in group A and 18.0% in group B (P = 0.003). The causes of procedure-related deaths for the whole group are shown in Table 3. The median time for procedure-related death in group A was 87 days (range 28-221 days) and in group B was 148.5 days (range 74-287 days). All but one of the procedure-related deaths in group A were secondary to infection.

**Discussion**

This report highlights important differences in the outcome of HLA-identical sibling SCT following two approaches to
Table 3  Patient outcome

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<th>(\beta)GVHD</th>
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*Infections post day 21.

L = limited; E = extensive; Pn = pneumonitis; IP = idiopathic pneumonitis; IPA = invasive pulmonary aspergillosis; CMV = cytomegalovirus; RSV = respiratory syncytial virus; ARDS = adult respiratory distress syndrome; EBV-LPD = Epstein–Barr virus-associated lymphoproliferative disorder; NE = not evaluable.

In vitro TCD, using either positive selection of CD34+ cells from PBSC or CAMPATH 1M treatment of bone marrow. Patients in both treatment groups received additional in vivo TCD, identical conditioning and no post-transplant immunosuppression. We found that allogeneic SCT using PBSC heavily depleted of T cells by immuno-magnetic selection of CD34+ cells, was associated with prolonged lymphocytopenia and an extremely high number of opportunistic infections leading to a high rate of procedure-related deaths. In contrast, allogeneic SCT using in vitro CAMPATH 1M treatment of bone marrow was associated with a lesser depletion of T cells, fewer infections and more rapid immune reconstitution. Both strategies were effective at preventing acute GVHD, and excluding those patients who received donor lymphocytes post transplant, at preventing extensive chronic GVHD. There was a low risk of graft rejection in both groups. Since the follow-up is relatively short and there are too few long-term survivors in group A, no conclusions can be made regarding the risk of relapse.

It seems likely that the overall determinant of clinical outcome in this report was degree of T cell depletion. There are five important qualifications to this statement. First, the type of graft differed in each group, such that patients in group A received PBSC and patients in group B received bone marrow. Thus, there may have been both qualitative (eg the number of CD34+ cells or CD34+CD38− lymphocyte progenitors)27 and qualitative differences (eg the balance between Th1 or Th2 T cells)28 that could have conceivably affected outcome. However, to our knowledge, there are no substantive clinical data to support the contention that these differences could account for such a poor outcome in the PBSC arm. Second, exact comparison of the degree of TCD for the two methods is difficult since following CD34+ cell selection nearly all the remaining T cells will be viable whereas this may not be the case following in vitro treatment with CAMPATH 1M.29 Indeed, antibody coated T cells may undergo further lysis when they encounter fresh complement following infusion. Furthermore, pre-transplant in vivo CAMPATH 1-H treatment could have resulted
Table 4  Infectious complicationsa

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*aInfections post day 21.
MRSA = methicillin-resistant Staphylococcus aureus; VZV = varicella zoster virus; HSV = herpes simplex virus; PF = para-influenza; IPA = invasive pulmonary aspergillosis.

Figure 1  Absolute lymphocyte counts post-transplant. Mean ± s.e.m.
absolute lymphocyte counts (%IOPF) post transplant are given for group
A (clear bars) and B (stipped bars). The number of patients in each group
for each time point are as follows: 1 month - A 4, B 22; 2 months - A 8, B 20; 3 months - A 6, B 19; 4 months - A 5, B 15. Significant differences between group A and B were observed at 2
months (P = 0.005), 3 months (P = 0.007) and 4 months (P < 0.05).

Figure 2  Overall survival. Group A shown as dotted line and group B
as solid line.

in further destruction of donor T cells in the recipient and
it is conceivable that there are differences between the two
modes of TCD in terms of the sensitivity of the remaining
T cells to in vivo lysis. It seems unlikely, however, that
these factors could account for the >2 log difference in T
cell dose observed. Third, although CD3 4 cell selection
removes most other cell types. CAMPATH 1M may be less
efficient at removing certain cells such as NK cells (our
unpublished observations) that could be of benefit in the
patient post transplant. However, there are few clinical data
available that clearly define the effect of changing the num-
ber of NK cells or other non-T cells in the graft upon the
post-transplant course. Fourth, this was not a randomised
study and 'clusters' can arise due to local or seasonal fac-
tors, which make an assessment of the reasons for dif-
fences between the two groups problematic. Finally, the
patients in the two groups may have differed in certain
characteristics that biased our evaluation of outcome.
Although there were some differences between the groups
in terms of the spectrum of diseases treated, group B had
more high risk patients and included three patients who had
received a previous transplant, suggesting any bias was
likely to favour the null hypothesis that there was no dif-
fence in outcome between the groups.

All of the above factors may have had the potential to
influence outcome, but one of the key differences between
the groups was the measured number of T cells adminis-
tered with the graft. A 'safe' threshold could be defined as
the minimum T cell dose that permits high rates of engraft-
ment, low rates of GVHD and low rates of serious infec-
tion. Any such level will be heavily influenced by patient
factors such as age or status, donor characteristics
including the degree of HLA disparity or CD3 4 cell dose,
and by other transplant factors such as the use of in vivo
TCD or post-transplant immunosuppression. For example,
there is abundant evidence that 'safe' T cell doses as
defined for TCD HLA-identical sibling SCTs are less safe
in the context of unrelated or mismatched transplants with
resulting high rates of graft failure or infection.12,30,31

Using this protocol, our data suggest that extensive TCD
of PBSC was associated with a very low risk of GVHD, high
rates of engraftment but severely compromised
immune reconstitution. There is little consistent infor-
mation regarding the 'safe' T cell threshold in terms of
immune competence of the recipient following HLA-identi-

cal sibling SCT. Recent studies indicate that infections
cause death in less than 10% of patients following standard T cell-replete SCT, with no difference between recipients of peripheral blood stem cells or bone marrow. In an early study of 31 patients, intensive TCD of bone marrow by an E-rosette technique and administration of a fixed T cell dose of 0.1 x 10^6 T cells/kg was associated with only three infective deaths. Low rates of infection were also observed in a recent study of 14 patients who received CD34+ enriched bone marrow cells and a mean T cell dose of 0.09 x 10^9/kg. For the most part, other studies in the HLA-identical sibling transplant setting suggest that lesser degrees of TCD (T cell dose > 0.1 x 10^6 T cells/kg) are associated with no major increase in the risk of opportunistic infection, although there are exceptions to this. The effect of additional in vivo TCD is unclear. In a retrospective analysis of two approaches, the addition of in vivo CAMPATH 1G to a protocol using in vitro TCD of the graft using CAMPATH 1H was not associated with higher rates of serious infection. Our report suggests that in adults, depletion of PBSC to 0.02 x 10^9 T cells/kg cells in combination with in vivo TCD using CAMPATH 1H results in prohibitively high rates of opportunistic infection following HLA-identical sibling SCT, that offsets any benefit in terms of GVHD reduction. Patients suffered considerable morbidity with more infections, more simultaneous infections and sub-optimal responses to antimicrobial therapy. Since initial immune reconstitution in adults is primarily dependent upon thymus-independent expansion of peripheral memory T cells from the graft, a major reduction in their number results in the loss of certain pathogen-specific immune responses. Excessive TCD, as reported here, appears to reduce the peripheral mature T cell pool below a critical threshold that ensures adequate resistance of the recipient to infection. Although, thymic output of naive T cells may eventually increase the T cell repertoire, its contribution is age-dependent and is likely to be delayed in adult patients. Thus, it is possible that younger patients may tolerate greater degrees of TCD without an unacceptable risk of infection.

One potential strategy to overcome the disadvantages of extensive TCD is the 'add-back' of T cells at a fixed dose at the time of transplant. It is possible that T cell doses lower than 1 x 10^6/kg could be administered safely following transplant if in vivo TCD using sequestration was omitted from the conditioning regimen. However, the optimal timing and dose of T-cell add-back post transplant are unknown at present but will be influenced by the anticipated risks of GVHD or relapse at any one point post transplant. Other patients who may benefit from early add-back of T cells are those at risk of CMV reactivation or those who have persistent uncontrolled infections. It is possible that specific markers of immune reconstitution, such as thymic output of naive T cells, may provide a better guide to patients who are most likely to benefit from such a strategy. An alternative approach might be to use in vitro TCD of PBSC using CAMPATH 1H, which is reported to be effective in permitting engraftment and preventing GVHD. In conclusion, we have reported the adverse effect of extensive in vitro TCD of PBSC upon TRM following HLA-identical sibling SCT. There was an unacceptably high risk of opportunistic infection in adult patients receiving CD34+ selected cells from peripheral blood and a mean T cell dose of 0.02 x 10^9 T cells/kg which resulted in a considerable morbidity and which was responsible for 85% of procedure-related deaths. It is possible that the use of additional in vivo TCD with CAMPATH 1H further reduced the effective T cell dose. These results suggest that consideration should be given to fixed dose donor T cell add-back in patients who have received PBSC grafts which are extensively (>5 log) T cell depleted or that measures taken to achieve in vivo TCD are omitted.

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References


T-cell depletion with Campath-1H ‘in the bag’ for matched related allogeneic peripheral blood stem cell transplantation is associated with reduced graft-versus-host disease, rapid immune constitution and improved survival

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Summary. We studied the outcome of 24 peripheral blood stem cell (PBSC) graft recipients, who were T-cell depleted (TCD) with either 20 mg (n = 14) or 10 mg (n = 10) Campath-1H in vitro, in comparison with a retrospective cohort of 23 unmanipulated (UM) PBSC recipients. While the neutrophil engraftment was similar, the platelet engraftment occurred earlier in the TCD group (d 11 vs 14). The incidence of acute and chronic graft-versus-host disease (GVHD) was 8-7% and 4-4% in the TCD group, respectively, compared with 47-7% and 56-3% in UM group (P < 0.001). In the TCD group, 5/6 chronic myeloid leukaemia (CML) and 4/18 non-CML patients relapsed (vs 0/6 and 3/17 in UM group, P = 0.06). All four molecular or cytogenetic relapses of CML were disease-free survivors following donor lymphocyte infusion. There was no difference in the incidence of serious infection between the TCD and UM groups and the lymphocyte recovery at 100 d was comparable. In the TCD cohort, the lymphocyte recovery was quicker in the 10 mg Campath-1H group. The non-relapse mortality (19.1% vs 66.3%) and 3 year survival (73.1 vs 19.2) were improved in the TCD group (P = 0.05). Thus elimination of late mortalities related to chronic GVHD and a rapid immune reconstitution, limiting either infection or relapse related deaths, contributed to an improved outcome following T-cell depletion with Campath-1H ‘in the bag’.

Keywords: Campath-1H, T-cell depletion, peripheral blood stem cell transplantation.

A major development in the field of allogeneic transplantation has been the use of growth-factor-mobilized peripheral blood stem cells (PBSC). The most consistent finding from studies on PBSC allografts has been a faster engraftment of both neutrophils and platelets, compared with bone marrow (BM) grafts (Blaise et al. 2000; Champlin et al. 2000; Bensinger et al. 2001; Korbling & Anderlini, 2001; Schmitz et al. 2002). Although this does not seem to improve the survival in standard-risk leukaemia (Schmitz et al. 2002) following sibling allogeneic stem cell transplantation (allo-SCT), there is a demonstrable impact on both survival and relapse in advanced leukaemias (Bensinger et al. 2001; Guardiola et al. 2002). However, these advantages of using a PBSC graft are negated by the increased incidence of graft-versus-host disease (GVHD). The incidence of chronic GVHD following PBSC allografts has varied between 46% and 67% in randomized studies, and a recent meta-analysis showed that the relative risk of developing extensive GVHD was significantly increased following PBSC (Cutler et al. 2001). The same meta-analysis also suggested an increase in the severity of acute GVHD with PBSC grafts, although this has been a less consistent finding in both randomized and non-randomized studies.

The challenge, therefore, lies in exploiting the engraftment and the antileukaemic potential of PBSC grafts and yet reducing the incidence and severity of GVHD. PBSC grafts

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contain about 10 times more lymphocytes than BM grafts (Ottinger et al., 1996; Steren et al., 2001). Some studies have attributed the increased incidence of GVHD to the higher number of T cells infused with PBSC grafts (Cutler et al., 2001), while others have correlated it with a high CD34+ cell count (Przepiorka et al., 2001; Zaucha et al., 2001).

T-cell depletion (TCD) has been the most effective method for the prevention of GVHD to date. However, following TCD of BM grafts, there has been an increased incidence of graft-failure, infections as a result of a delay in immune reconstitution and an increase in the incidence of relapse (Hale et al., 1998; Lowdell et al., 1998; Papadopoulos et al., 1998; Small et al., 1999). Few studies have explored the outcome of TCD-PBSC grafts, particularly in terms of immune reconstitution (Martinez et al., 1999; Hale et al., 2000; Barbe et al., 2001).

The method of T-cell depletion has been widely variable, ranging from physical methods (Papadopoulos et al., 1998) and the use of monoclonal antibodies (Hale et al., 1998) to column-based immunomagnetic selection of specific cell populations (Martinez et al., 1999). Campath antibodies have long been used both in vivo and in vitro for T-cell depletion. Campath-1M in vitro resulted in graft failure in 16% of patients, but this risk was reduced by the use of Campath-1G in vivo as a part of conditioning treatment (Hale et al., 1998). In vitro Campath-1G has been used alone for PBSC grafts and patients have shown an improved survival at 6 months (Barbe et al., 2001), when compared with unmanipulated graft patients. Campath-1H is the humanized form of this antibody, which has recently been licensed by the Food and Drugs Administration (FDA) for use in chronic lymphocytic leukaemia.

Our centre was one of the first to adopt PBSC as the stem cell source in matched related transplantation. We observed both rapid engraftment and a low incidence of relapse. However, owing to a high incidence of late chronic GVHD observed in these patients, in June 1998 we elected to T-cell deplete PBSC grafts using Campath-1H in vitro. We report the outcome following T-cell depletion of PBSC grafts from matched related donors using Campath-1H antibody in vitro and compare this with a retrospective cohort of patients receiving unmanipulated (UM) PBSC grafts.

PATIENTS AND METHODS

We evaluated 47 consecutive allograft recipients who were treated in the Bone Marrow Transplant (BMT) Unit at Birmingham Heartlands Hospital between June 1996 and September 2001. Twenty-four patients received a T cell-depleted PBSC graft (TCD) from July 1998 to September 2001 and were compared with a retrospective cohort of 23 patients who received an unmanipulated PBSC graft (UM) from June 1996 to June 1998. All were transplanted for haematological malignancies.

Conditioning treatment

Conditioning regimens consisted of either cyclophosphamide 60 mg/kg for 2 d (n = 32) or etoposide 60 mg/kg single dose [for patients with acute lymphocytic leukaemia (ALL) only, n = 12] and fractionated total body irradiation (TBI) at 1440 cGy in eight doses. Patients not receiving TBI received cyclophosphamide at the above dose and busulphan 16 mg/kg over 4 d in four daily divided doses (n = 3).

PBSC mobilization and apheresis. All the donors were fully matched for both human leucocyte antigens (HLA) class I (A, B, Cw) and class II (DRB1, DP and DQ) using the polymerase chain reaction with sequence-specific primers (PCR-SSP). The donors were treated with lenograstim (Chugai pharmaceuticals, UK) at a dose of 10 μg/kg subcutaneously in the afternoon for 4 d. The stem cell apheresis was carried out using a Baxter CS 3000 continuous-flow automated cell separator from d 5 onwards for a maximum of 3 d. The donors received lenograstim at the same dose in the evening each day after completion of the apheresis. The procedure was carried out using peripheral venous access, and a femoral catheter was used in donors with poor peripheral access. For the TCD group, the target CD34+ cell dose was 5 × 10^6/kg of patient body weight. The excess amount, if any, was stored as CD3+ cell aliquots for future use as donor lymphocyte infusions (DLI).

T-cell depletion

T-cell depletion was carried out with Campath-1H in vitro. The apheresis products were stored for a maximum of 48 h and were pooled to a maximum volume of 500 ml. Campath-1H 20 mg (n = 14) or 10 mg (n = 10) was added to the final product. These were then incubated at 25°C in an agitator for 30 min. The cells were immediately infused to the patient over 30–60 min following premedication with intravenous methylprednisolone 1 mg/kg, paracetamol and chlorpheniramine 1 h before the infusion. Samples from pooled apheresis products were obtained both prior to and following incubation with Campath-1H. These were evaluated for CD34+ and CD3+ cell counts. Assays were carried out using a Becton Dickinson FACSCalibur flow cytometer. CD34 analysis was performed using a dual platform technique according to the International Society for Hemotherapy and Graft Engineering (ISHAGE) protocol using anti-CD34 and anti-CD45 antibodies (Sutherland et al., 1996). CD3+ T-cell analysis recorded CD3 events staining with anti-CD3 as a proportion of total nucleated cells staining with anti-CD45. All the monoclonal antibodies were obtained from Becton Dickinson (Oxford, UK).

GVHD prophylaxis

Patients transplanted with TCD-PBSC received intravenous cyclosporin A at 3 mg/kg in two divided doses from d −1 until they were able to tolerate oral cyclosporine. Oral cyclosporine was intended to continue to d + 180 post transplantation in the original protocol, with subsequent tapering. However, this was modified after the first five patients to continue to d + 100 only. Cyclosporine was continued beyond this period only in patients with GVHD. The dose of cyclosporin A was titrated to maintain plasma levels between 150 and 250 ng/l.
In the group of patients receiving UM-PBSC, cyclosporin A was continued at an identical dosage schedule for 6 months and short-course methotrexate was added at 15 mg/m^2 on d 1 and 10 mg/m^2 on d 3, 6 and 11 post transplantation.

Donor lymphocyte infusion
DLI was used following TCD-PBSC transplants for molecular, cytogenetic or haematological relapse of chronic myeloid leukaemia (CML) in an escalating dose schedule. The initial CD3⁺ cell dose was 1 × 10⁸/kg and this was followed by an increment of half a log every 3–4 months until cytogenetic and/or molecular remission was documented. The maximum dose used was 1 × 10⁹/kg CD3⁺ cells.

Disease status was evaluated on BM aspirate specimens at 3 months on all patients. Peripheral blood samples of patients transplanted for CML were obtained every 6 weeks for cytogenetic and molecular evaluations. Cytogenetic relapse was defined as sustained presence of t(9:22) on two separate occasions 4 weeks apart. Molecular relapse was defined as the demonstration of bcr-abl fusion mRNA transcripts on two separate occasions 4 weeks apart in the absence of cytogenetic or haematological evidence of CML. Once evident in peripheral blood, these were confirmed on BM samples.

Supportive care
All the patients were nursed in single rooms with high-efficiency particulate air (HEPA) filters. Anti-microbial prophylaxis consisted of oral fluconazole, ciprofloxacin and oral aciclovir from the beginning of conditioning treatment until engraftment. Oral cotrimoxazole was initiated when the neutrophil count was greater than 1 × 10⁹/L. Metronidazole 400 mg thrice daily, was started between the day of transplant to engraftment for anaerobic decontamination as a part of GVHD prophylaxis from January 1998.

Cytomegalovirus (CMV) sero-positive recipients or those receiving grafts from CMV sero-positive donors were screened for CMV reactivation by a non-nested polymerase chain reaction (PCR)-based assay (Ampliprime; Roche, Lewes, East Sussex, UK) on whole blood collected in ethylenediamine tetra-acetic acid (EDTA) (Chakrabarti et al, 2002a). The patients were screened every week from transplantation to 100 d post transplantation. Generally those patients who had CMV infection before 100 d or had GVHD were screened for 180 d or more. The patients received antiviral therapy if two consecutive PCR assay results were positive. CMV sero-negative patients receiving grafts from CMV sero-negative donors received CMV-negative blood products. Surveillance for other viruses was carried out on stool, urine and throat samples to 180 d post transplant.

Chimaerism. For TCD-PBSC recipients, chimaerism was analysed at 30, 90 and 120 d post transplantation and every 3 months thereafter for the first 2 years. This was carried out by fluorescent in-situ hybridization (FISH) with Y chromosome probes for sex-mismatched transplants. For sex-matched transplants, the peripheral blood and BM were assessed for donor chimaerism using microsatellite markers (Chakrabarti et al, 2001a). For the UM-PBSC cohort, chimaerism data were available for patients transplanted after 1997, at least once in the first year after transplantation.

Immune reconstitution
The absolute lymphocyte count (ALC) was noted at least once every 4 weeks following discharge for the first 6 months. The CD4¹ and CD8¹ T-cell counts (from August 1999) were measured every 6–8 weeks post transplant as previously described (Chakrabarti et al, 2002b).

Definitions and statistical methods. Disease status was defined as advanced if: acute leukaemia was beyond complete remission (CR)-1 or refractory, CML was beyond chronic phase (CP1), myeloma was beyond first plateau or poorly responsive to first-line therapy, myelodysplastic syndrome (MDS) was of the subtype refractory anaemia with excess of blasts (RAEB) or refractory anaemia with excess of blasts in transformation (RAEB-t) by the French–American–British (FAB) classification, or if adverse cytogenetics were detected at diagnosis in patients in CR1. Engraftment was defined as neutrophil recovery to more than 0·5 × 10⁹/L and unsupported platelet count of 20 × 10⁹/L. Acute and chronic GVHD were defined according to standard criteria (Glucksberg et al, 1974; Shulman et al, 1980). Acute GVHD could be evaluated in all patients beyond d 0 up to d 100 and chronic GVHD in survivors beyond 100 d. Non-relapse mortality (NRM) was defined as death from any cause in patients in remission.

Univariate P-values and odds ratios were calculated from 2 × 2 contingency tables using Fisher's exact test where appropriate. The continuous variables were compared using Mann–Whitney's non-parametric method. Cumulative probabilities of engraftment, acute and chronic GVHD, relapse, overall survival, and non-relapse mortality were analysed with censuring for competing risk factors as appropriate, and the difference between the groups was compared using the log rank χ² test. The Kaplan–Meier method was used for estimating overall survival. The effect of the variables on GVHD, relapse and NRM were tested in a Cox-regression model. The variables examined were age, donor sex, disease type (standard vs advanced), CMV sero-status, graft composition (CD34⁺ cells) and graft manipulation (TCD vs UM). The variables were analysed in a univariate model and the variables with P-value less than 0·10 were entered in the multivariate model.

RESULTS
Patient characteristics
The patient characteristics are detailed in Table I. Twenty-three patients received an UM graft and 24 received a TCD graft. There was no difference in the age, sex or underlying disease between the groups. There was a slight preponderance of advanced disease and female donors in the UM-PBSC group.
Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>T cell-depleted (n = 24)</th>
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<th>P-value</th>
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<tr>
<td>Age median (range)</td>
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<td>11/3</td>
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<tr>
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<td>Disease</td>
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<tr>
<td>Graft Cd34^+ cells/kg</td>
<td>Med. (range)</td>
<td>5.2 (2.5–9.7)</td>
<td>4.4 (1.5–6.4)</td>
</tr>
</tbody>
</table>

Graft composition
The CD34⁺ cell dose in the TCD-PBSC group was 5.2 × 10⁶/kg. This was similar to the UM-PBSC group. The median CD3 dose for the TCD group was 292 × 10⁶/kg (range 37–519). The depletion of CD34⁺ cells achieved after incubation with Campath-1H was less than one log in all cases, varying between 0.1 and 0.8 log. There was no difference in the in vitro depletion between the use of 10 mg or 20 mg Campath-1H.

Engraftment
The neutrophil engraftment occurred at a median of 14 d [95% confidence interval (CI): 12.6–15.4] in the TCD group and 13 d (95% CI 11.8–14.2) in the UM group (P = 0.8, Fig 1A). One patient in the UM group succumbed to a fulminating infection before engraftment. However, the platelet engraftment was faster in the TCD group (median 11 d [95% CI 9.8–12.2] vs 14 d [95% CI 13.1–14.9] in UM-PBSC, P = 0.00040 [Fig 1B]).

There was no secondary graft failure in the UM group and there was one late graft failure in the TCD group. This 33-year-old woman was transplanted for ALL in the first remission and had a prompt engraftment and an uneventful course until d 95. The patient’s blood counts declined progressively over the next 2 weeks with reversal of chimaerism to recipient type as measured by FISH technique. The BM was aplastic and there was no evidence of leukemia. She failed to engraft following conditioning with antithymocyte globulin and received further conditioning with fludarabine and melphalan. Although there was evidence of engraftment at d + 12, she succumbed to viral pneumonitis.

Acute GVHD
Ten UM-PBSC recipients developed acute GVHD of grade 2–4. Five of them had grade 3–4 GVHD. On the other hand, only two patients in the TCD group developed grade 2 GVHD, which was limited to the skin. Three other patients developed a mild non-specific rash in the extremities between 10 and 20 d post transplantation that resolved without treatment. The cumulative probability of grade 2–4 GVHD was significantly lower in the TCD group (8.7%, 95% CI 0–20) when compared with the UM group (47.7%, 95% CI 26–69) (P = 0.003, Fig 2A).

Chronic GVHD
Ten of the 18 patients who could be evaluated beyond 100 d developed chronic GVHD in the UM group, six of whom had extensive chronic GVHD. Only one of the 23 patients who could be evaluated developed mild oral chronic GVHD in the TCD group. The cumulative probability of chronic GVHD was 4.2% (95% CI 0–12.6) in the
Relapse

Nine out of the 24 patients receiving TCD-PBSC relapsed with a cumulative probability of 48.7% (95% CI 24.4–73.2), whereas 3/23 patients relapsed in the UM-PBSC group (cumulative probability, 15.4% at 2 years, 95% CI 0–35.2) (P = 0.06, Fig 3). However, only 2/10 patients receiving grafts treated with Campath-1H 10 mg relapsed compared with 7/14 patients receiving grafts treated with Campath-1H 20 mg (P = 0.14). None of the six CML patients relapsed in the UM group, whereas 5/6 relapsed in the TCD group. However, 4/5 relapsed patients suffered only either molecular or cytogenetic relapse, and one patient transplanted for accelerated-phase CML had haematological relapse.

There was no difference in the relapse rate of non-CML patients, with three and four patients with acute leukaemia relapsing in UM and TCD group respectively. Complete donor chimaerism was observed in the majority of the patients with acute leukaemia, and in all of the six CML patients in the UM group, whereas all the CML patients in the TCD group had persistent mixed chimaerism prior to relapse (45–90% donor cells). Following DLI conversion to full donor chimaerism was observed in all the responders.

On multivariate analysis, graft manipulation was the only factor that positively influenced acute GVHD (P = 0.04, relative risk 0.98, 95% CI 0.92–0.94) or chronic GVHD (P = 0.001, relative risk 0.07, 95% CI 0.008–0.6).

**Disease-free survival (DFS) and GVHD following DLI**

At the time of writing, all of the four patients with CML who were documented to have cytogenetic and/or molecular relapse following TCD-PBSC were in remission following escalation of the DLI dose. The only patient who had a haematological relapse was transplanted in the accelerated phase of CML. He succumbed to post-DLI aplasia. Only one of the four patients with acute leukaemia who relapsed was a disease-free survivor at 12 months follow-up. Two patients succumbed to relapsed disease and another was still on treatment at the time of writing.

Disease control with DLI was at the cost of only a slight rise in the overall incidence of GVHD. Four patients developed grade 2–3 GVHD following DLI in the TCD group. In three of them, it followed bulk dose DLI (1 × 10^7/kg) for relapsed AML or ALL, and one patient was a long-term
survivor. Only one of the four patients receiving an escalating dose DLI for CML developed grade 2 GVHD which responded to steroids.

The overall probability of GVHD in the DLI in the TCD group was 25% (95% CI 9–41, P = 0.05) and in disease-free survivors was 16.7% (95% CI 2.7–30.7, P = 0.02) which was still significantly lower than the UM group.

**Immune reconstitution**

The recovery of the ALC was slower in the TCD group at 30 d (mean ± standard deviation [SD]/mm³, 410 ± 160 vs 910 ± 441 in the UM group, P = 0.002). However, this was comparable at 100 d (mean ± SD/mm³, 1029 ± 654 vs 1082 ± 612 in the UM group, P = 0.8).

In the TCD group, the ALC recovery was faster at both 30 d (mean ± SD, 0.475 ± 0.152 × 10⁹/l vs 0.364 ± 0.160 × 10⁹/l, P = 0.09) and 100 d (1.350 ± 0.707 × 10⁹/l vs 0.807 ± 0.438 × 10⁹/l, P = 0.03) in patients receiving grafts treated with 10 mg Campath-1H, compared with those receiving grafts treated with 20 mg of the antibody.

The recovery of CD4⁺ T cells was studied in the TCD group only. The CD4⁺ count at 100 d for the patients receiving 10 mg Campath was significantly higher than those receiving 20 mg Campath (mean ± SD, 0.281 ± 0.089 × 10⁹/l vs 0.161 ± 0.086 × 10⁹/l, P = 0.01). Among patients who received 20 mg Campath in vitro, the median time to achieve a CD4⁺ count of 0.2 × 10⁹/l was 7 months, compared with 3 months for those receiving 10 mg Campath-1H.

**Infections**

Three out of the 10 UM patients at risk of CMV disease and four out of the 11 TCD patients at risk of CMV disease showed reactivation of the CMV respectively (Table I). None in the UM-PBSC group had CMV disease. One patient in the TCD-PBSC group developed CMV colitis at 60 d post transplant, following a failure to comply with weekly CMV monitoring. This, however, resolved following antiviral therapy. Other serious infections were documented in seven UM-PBSC recipients (bacterial sepsis, three; resistant herpes simplex virus, two; aspergillus, one; Clostridium difficile, one) and in four TCD-PBSC recipients (community respiratory virus, two; suspected fungus, one; suspected pneumocystis pneumonia, one).

**Non-relapse mortality**

Only four patients died from transplant-related causes in the TCD group at a median follow-up of 24 months (range 6–45 months), compared with 10 deaths in the UM group. The probability of NRM was 19.1% (95% CI 1.9–36.3) compared with 66.3% (95% CI 40.7–91.9) in the UM group (P = 0.05, Fig 4). Half of the mortality in the UM group was attributable to GVHD, whereas there was no GVHD-related mortality in the TCD group (Table II). It should be noted that the NRM in the UM-PBSC recipients at 24 months was only 24.1% (Fig 4). However, the five deaths that occurred in the UM group beyond 2 years were all attributable to chronic GVHD or complications related to its therapy.

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Table II. Causes of mortality.

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<td>Fungal</td>
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</tr>
<tr>
<td>Relapse</td>
<td>1</td>
</tr>
<tr>
<td>Others</td>
<td>1</td>
</tr>
<tr>
<td>TTP</td>
<td>1</td>
</tr>
<tr>
<td>Pneumonitis</td>
<td>0</td>
</tr>
</tbody>
</table>

TTP, thrombotic thrombocytopenic purpura.

On multivariate analysis, graft manipulation (P = 0.06, relative risk 0.3, 95% CI 0.1–1.06) and a higher CD34⁺ cell dose (P = 0.06, relative risk 0.7, 95% CI 0.5–1.08) had a trend towards a positive influence on NRM.

**Overall survival**

The OS at 2 years was 63.4% (95% CI 41.7–85.1) for the UM group and 73.1% (95% CI 54.5–91.7) for the TCD group (Fig 5). As mentioned in the previous section, five deaths occurred in the UM group after 24 months and none occurred in the TCD group beyond this period. This conferred a significant survival advantage to the TCD group with an actuarial survival at 3 years of 73.1% (95% CI 41.7–85.1), compared with 19% (95% CI 2.3–36) for the UM group (P = 0.05). When stratified by age, there was no difference in the survival or NRM between patients aged above or below 35 years.
Fig 5. The overall survival of UM and TCD patients following transplantation.

DISCUSSION

T-cell depletion is the most effective method for prevention of GVHD, but this is often at the cost of an increase in graft rejection, relapse and poor immune reconstitution with consequent infections. In this study, we have attempted to strike a balance between these factors by optimizing the dose of Campath-1H 'in the bag'. After analysing the first 14 patients receiving Campath-1H 20 mg 'in the bag', we reduced the dose of Campath added to the graft to 10 mg because of an extremely low incidence of GVHD, and in order to explore the possibility of improving immune reconstitution and reducing relapse risk. The most notable difference was noted in the prompt immune reconstitution, which occurred without an increase in GVHD, and a trend towards a reduction in the relapse risk was also observed.

Graft failure and rejection are major concerns following T-cell depletion. All the previous studies reporting successful engraftment have incorporated additional agents such as antibodies, lymphoid irradiation or thiopeta in the conditioning regimen (Bunjes et al. 1995; Hale et al. 1998; Papadopoulos et al. 1998; Barge et al. 2001). We have demonstrated sustained engraftment in all but one patient without the use of additional agents. It is possible that additional immunosuppression is not necessary if either the dose of TBI is 1 4 Gy or if there could have been an extended effect of the Campath that was infused with the graft on host lymphocytes. In addition, the high CD34+ cell dose in the TCD grafts may also have facilitated engraftment. The neutrophil engraftment was similar in the two groups, while the platelet engraftment was faster in the TCD group. Although there was a trend towards a correlation with higher CD34+ cell count (data not shown), the only other explanation for this could be the absence of methotrexate treatment as a GVHD prophylaxis in the TCD group.

The incidence of acute and chronic GVHD following PBSC transplantation in most previous studies has varied between 27% and 52% and 35–67%, respectively, and the survival at 2 years has varied between 42% and 65% (Blaise et al. 2000; Champlin et al. 2000; Bensinger et al. 2001; Korbling & Anderlini, 2001; Schmitz et al. 2002). Our experience with the use of PBSC grafts was very similar, with the cumulative incidence of acute and chronic GVHD being 47.7% and 56% and the disease-free survival at 2 years being 63%. There was an extremely low incidence of relapse when taking account of the fact that 40% of the patients in the UM-PBSC group had more advanced disease. This favourable outcome at 2 years was offset by late mortality due to severe chronic GVHD and its therapy. The survival of our UM-PBSC cohort beyond 2 years was much lower than those reported in randomized studies and this may have exaggerated the survival advantage of the TCD group. While, this could be due to the more high-risk patients in this group, the small sample size, the improvement in supportive care since 1998 and the fact that this is not a randomized comparison, there are a few points worth noting. Recent publications suggest that on longer follow-up, GVHD following PBSC transplants is both more protracted and difficult to treat (Flowers et al. 2002). Another study reported only a 15% survival in PBSC graft recipients with chronic extensive GVHD (Pezzi et al. 2001). All the randomized studies to date have reported only on the 2-year survival of patients, and the long-term follow-up of these cohorts is required to evaluate the impact of chronic GVHD.

The use of T-cell-depleted BM grafts has been associated with a low incidence of both acute and chronic GVHD (Bunjes et al. 1995; Hale et al. 1998; Papadopoulos et al. 1998). Studies exploring the outcome of T-cell depletion of PBSC grafts are low in number. In one study, the use of Campath-1G-treated PBSC grafts was associated with 18% and 23% acute and chronic GVHD respectively (Barge et al. 2001). Another multicentre survey reported the incidence of acute and chronic GVHD to be 4% and 24% respectively (Hale et al. 2000). The incidence of acute (8.7%) and chronic (4.4%) GVHD in our cohort was much lower than in previous reports. This reduction in chronic GVHD translated into an improved survival, in the absence of an increase in infection or relapse-related deaths.

While the incidence of relapse was markedly increased in CML following T-cell depletion, the earlier detection of either a molecular or cytogenetic relapse and the use of an escalating dose of DLI enabled the induction of a second remission in all but one patient. The only exception was a patient with accelerated-phase CML in haematological relapse. Thus, the DFS for patients with CML at the time of writing was not different between the two groups maintaining the lower incidence of GVHD. Similar findings have been reported in other studies on TCD transplants in CML (Drobyski et al. 1999; Schn et al. 1999). Thus, this approach could be suitable for patients with CML at a higher risk of GVHD, if effective molecular or cytogenetic monitoring could be ensured to detect early relapse. There was no increase in the relapse rate of acute leukaemias in the TCD group. It is worth noting that the majority of the patients in the TCD group were in first remission and that previous studies have suggested that T-cell depletion does not increase the
risk of relapse in AML in first remission in the absence of other poor prognostic features (Bunjes et al., 1995; Hale et al., 1998; Papadopoulos et al., 1998).

Two previous studies have investigated immune reconstitution following the use of Campath in vitro with BM grafts. The use of both Campath-1M and -1G was associated with a slower recovery of CD4+ cells (Lowdell et al., 1998) and a high incidence of life-threatening viral and non-viral infections (Davison et al., 2000). PBSC grafts have been associated with a more rapid immune reconstitution (Bensinger et al., 2001; Storok et al., 2001). There are limited data on the immune reconstitution following T-cell-depleted PBSCT. One study on CD34+ selected PBSCT transplants demonstrated a delayed reconstitution of T-cell subsets in the first 8 months after transplant (Martinez et al., 1999). An important finding in our study was the significantly different kinetics of immune recovery noted between the two doses of Campath-1H. While the use of 20 mg Campath-1H was associated with the recovery of CD4+ T cells and ALC at a slow but reasonable pace, especially when considering that the grafts were T-cell depleted, the reduction of the dose to 10 mg resulted in prompter recovery of both these parameters. Although the follow-up was shorter for this group of patients, it is possible that the improved immune reconstitution may also have had a favourable impact on the relapse risk (Powles et al., 1998; Kumar et al., 2001).

The lower NRM in our cohort was contributed to not only by reduction in GVHD, but also by the effective control of viral infections by the pre-emptive interventions for CMV, respiratory viruses and adenoviruses (Chakrabarti et al., 2001b, 2002a, b), and a rapid recovery of the ALC and CD4+ subset. The importance of lymphocyte recovery in the outcome of viral infections and survival has been highlighted in previous studies (Bensinger et al., 1993; Pavletic et al., 1998; Chakrabarti et al., 2002b). In addition, a higher CD34+ cell dose has been reported to be associated with a lower NRM (Bittencourt et al., 2002), with a similar trend noted in our study.

The majority of Campath antibody activity used by the methods described would have to take place in vivo by antibody-dependent cell-mediated cytoxicity (ADCC) and thus the in vitro T-cell depletion is not reflective of the true extent of depletion. Approximately, 5 mg Campath should be required for T-cell depletion when added to an average PBSC collection containing 3 x 10^6 CD34+ cells, but a much larger dose is used in clinical practice because the antibody binds the CD52 antigens with low affinity and, thus, the amount of free or circulating antibody would be widely variable and difficult to predict. It is, therefore, difficult to ascertain the minimum dose of Campath necessary for optimum T-cell depletion by in vitro experiments. This can be best answered through clinical trials. The reduction of the dose of Campath-1H to 10 mg was associated with an improved immune recovery without an increase in GVHD. This could be due to a less extensive T-cell depletion. The activity of Campath antibodies extends beyond T cells to dendritic cells. It is possible that their effect on dendritic cells is responsible for the prevention of GVHD when only a partial T-cell depletion was observed (Klangsinirikul et al., 2002). The other possibility is that the free antibody remaining in the circulation is likely to be higher if a higher dose is used. Campath-1H has been shown to have a prolonged half-life, which might interfere with immune recovery (Rebello et al., 2002).

While unmanipulated PBSC is the preferred option for advanced stage leukemias, measures to reduce chronic GVHD need to be optimized. For advanced diseases, the risk of GVHD can be significantly reduced without an increase in leukaemic relapse or compromise of the immune recovery, by the judicious use of T-cell depletion. In our study, Campath-1H 'in the bag' was effective in achieving this goal following matched related transplants. The use of 10 mg Campath-1H seemed to be the more pragmatic approach for matched related grafts, with a better reconstitution of cellular immunity observed, which might translate to a lower relapse risk even in patients with more advanced disease. This theory needs to be explored in larger studies. In fact, a multicentre randomized study is being initiated in the UK to compare unmanipulated and Campath-1H-treated grafts in patients with AML in first remission.

ACKNOWLEDGMENTS

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Camppath-1H in the Bag for T-cell Depletion


Lymphocyte reconstitution after allogeneic blood stem cell transplantation for hematologic malignancies. Bone Marrow Transplantation, 21, 33–41.


ALEMTUZUMAB IN VIVO

Alemtuzumab is now commercially available and over the last 6 years has replaced Campath-1G for stem cell transplantaion. It is generally used in the same manner as the previous rat antibody. In addition, a novel protocol was developed for a nonmyeloablative conditioning regimen: alemtuzumab was combined with fludarabine and melphalan to facilitate engraftment along with standard GVHD prophylaxis. This protocol was associated with a reduced incidence of GVHD, which is an important cause of mortality following other nonmyeloablative protocols. Amidst this excitement, we noted with dismay that the patients undergoing nonmyeloablative transplantation with this regimen were experiencing a far greater incidence of cytomegalovirus (CMV) reactivation and other virus infections.14.6

We initially compared the outcomes of transplant recipients after the nonmyeloablative regimen with those undergoing

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doi:10.1016/j.transproceed.2004.05.067
full-intensity conditioning before receiving grafts that had been T-cell-depleted with alemtuzumab in vitro. There was a significant difference in the onset and the overall incidence of viral infections between the groups: the former group experienced an earlier onset and much higher incidence of infection.\textsuperscript{2} We also noted slower immune recovery among the nonmyeloablative group.

We subsequently analyzed the viral surveillance of all patients including those receiving alemtuzumab either in vivo or in vitro, irrespective of the intensity of the conditioning regimen. CMV respiratory and adenovirus infections were significantly increased following the use of alemtuzumab in vivo, irrespective of the conditioning regimen.\textsuperscript{2-6} Additional patients from two other UK centers were also evaluated for these viral infections. A similar trend was noted in the multicenter evaluation of nonmyeloablative transplant recipients confirming that our initial findings were not due to a center-effect or a chance finding.\textsuperscript{7} These observations led to pharmacokinetic studies on alemtuzumab. Campath-1G has an estimated half-life of about 13 hours but alemtuzumab, of 15 to 21 days, suggesting that biologically active antibody may persist for several months.\textsuperscript{8} Thus the concentration of the humanized antibody when used in vivo in the dose of 100 mg over 5 days was higher than was necessary for in vivo T-cell depletion at the time of stem cell infusion, persisting at potentially lympholytic levels for months posttransplant. This persistence probably delayed lymphocyte recovery and increased the risk of viral infections, lending credence to the hypothesis that alemtuzumab (rather than other elements of the conditioning regimens) was responsible for the increased viral infections. Subsequently, the nonmyeloablative protocol was modified to explore reduction of the alemtuzumab dose to reduce this complication.

**ALEMTUZUMAB IN VITRO**

For full-intensity conditioning, we have used a protocol of T-cell depletion with alemtuzumab in vitro, similar to that reported by Cape Town.\textsuperscript{9} We started with a dose of 20 mg alemtuzumab. Although the problems of immune reconstitution and viral infections were reduced with this protocol, we further reduced the alemtuzumab dose, because there was virtually no GVHD with this in-vitro dose of alemtuzumab. The dose reduction was aimed at further improving immune reconstitution and reducing the relapse risk. Indeed, the immune reconstitution was significantly improved and early data suggest that the relapse risk might be lower. This was achieved without any increase in the incidence of GVHD. Viral infections were also less common. Because of the lower total dose of antibody, the blood concentration was expected to fall below lympholytic levels much more quickly.\textsuperscript{9}

Our studies have demonstrated that alemtuzumab, as currently used in vivo, decreases the incidence of GVHD at the cost of poor immune reconstitution and increased infectious complications. Subsequent pharmacokinetic studies have substantiated the hypothesis that longer persistence of high levels of alemtuzumab is responsible for the untoward effects. The aim to reduce GVHD is successfully achieved with alemtuzumab in vitro with less impairment of immune recovery and fewer resultant complications. Although this might be achievable in the conventional allograft setting, the few patients, who have been treated by reduced intensity conditioning together with in vitro T-cell depletion, have had difficulty achieving durable engraftment.\textsuperscript{10}

**FUTURE DIRECTIONS**

The longer half-life of human compared with rodent monoclonal antibodies is usually perceived to be an advantage. However, alemtuzumab treatment, as we have prescribed it, prevents rejection and GVHD in stem cell transplantation has resulted in antibody persistence beyond an optimal window leading to delayed immune reconstitution. The original rat antibody Campath-1G appears to have the perfect characteristics for this purpose, but regrettably is not available commercially. Alternative, modified dosing schedules may overcome this problem.

Alemtuzumab therapy, as currently practiced, may still have a role for nonmalignant disorders, where durable mixed chimerism without GVHD is the ideal outcome. Higher doses of alemtuzumab in vivo might still be necessary for mismatched grafts. T-cell depletion in the setting of conventional sibling allografts can be achieved successfully using low doses of alemtuzumab in vitro (10 mg) without compromising immune reconstitution. A higher dose in vitro (20 mg) might suffice for matched unrelated grafts. For mismatched unrelated grafts, further host immunosuppression might be achieved with fludarabine without resorting to in vivo alemtuzumab. Thus our cumulative experience with alemtuzumab suggests that better results might be achieved by tailoring the dose and mode of the use of this reagent to match the clinical situation. Further studies are needed to optimize the dose of alemtuzumab in vivo and in vitro, relevant to the type of conditioning and the graft.

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Prevention of Graft-Versus-Host Disease in Allogeneic Bone Marrow Transplantation for Leukemia by T Cell Depletion In Vitro Prior to Transplantation


THE ACUTE, and to a lesser degree, the chronic, forms of graft-v-host disease (GVHD) represent one of the major obstacles to successful bone marrow transplantation (BMT), since neither adequate preventive measures nor effective therapeutic modalities are known to date. Since GVHD results from interaction between mature donor T lymphocytes and recipient cells capable of stimulating the donor's immunocompetent cells, we have attempted to prevent GVHD by in vitro removal of mature T lymphocytes prior to transplantation. The present report represents a summary of our initial experience using three approaches for the depletion of T lymphocytes. Our data suggest that GVHD may be completely prevented by adequate removal of T lymphocytes from pretransplant marrow in patients undergoing BMT from HLA-matched sibling donors.

PATIENTS AND METHODS

A total of 21 consecutive leukemia patients, including 11 poor-risk patients with resistant disease in relapse or partial remission, underwent BMTs with T cell-depleted marrow obtained from HLA-A, B, DR-compatible matched sibling donors, starting in early 1983. Pertinent details are described in Table 1. Most patients were treated with cyclosporin 60 mg/kg on two consecutive days and two intrathoracic methotrexate injections for GCS prophylaxis followed by 2,200 rad whole body irradiation given in six fractions over three to six days from a linear accelerator at approximately 25 rad/min. T cell depletion was carried out using one of the following methods. In all cases, mononuclear-rich plasma was obtained after mixing heparinized marrow with Volds (betastrach) at a final concentration of 0.66% for one hour at room temperature by removal of the RBC sediment. Protocol 1: Ficol-Hypaque (FH) enrichment of stem cells, followed by separation of T cells rosetting with neuraminidase-treated sheep RBCs (E-RFC) over a second FH gradient. Protocol 2: Enrichment of stem cells by mixing marrow cells with soybean agglutinin (2 mg/mL) and separation of agglutinating cells over a 5% bovine serum albumin gradient. This procedure was previously described. Protocol 3: Treatment of marrow mononuclear cells (50 to 100 x 10^6/mL) reconstituted in buffered saline supplemented with Ca^++ and Mg^++ with a mononuclear rat-anti-human lymphocyte antibody (CAMPATH-1), originally described by Hale et al., at 100 µg/mL for 30 minutes, followed by addition of autologous donors' fresh serum as a source of complement for an additional 30 to 45 minutes in a 37°C bath. In all protocols, T cell removal was confirmed by studying E-RFC and colony formation (CFU-GM) before and after the procedure. None of the patients, received posttransplant immunosuppressive therapy.

RESULTS AND CONCLUSIONS

These data are based on a small and nonhomogenous group of patients (six in protocol I, five in protocol II, and ten in protocol III) including poor-risk patients with relapse or resistant disease, and therefore no firm conclusions can be reached on the role of T cell depletion in the overall survival and success rate following BMT. Nevertheless, several conclusions are noted: (1) All three protocols used are efficient for depletion of immunocompetent cells.

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### Table 1.

<table>
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<th>Case/Age</th>
<th>Diagnosis and Condition</th>
<th>Protocol</th>
<th>Asp. $\times 10^8$/kg</th>
<th>Final $\times 10^8$/kg</th>
<th>NE-RFC*</th>
<th>C/S</th>
<th>C/S +</th>
<th>Day of Last Patient Transplant</th>
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<td>3.60</td>
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<td>4.00</td>
<td>0.64</td>
<td>11.00/0.000</td>
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<td>3.00</td>
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<td>8.30/0.040</td>
<td>16</td>
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</table>

### CNS and systemic relapses
| F/11     | ALL, 2nd CR            | III      | 7.60                   | 1.29                   | 6.10/0.000  | 24   | 28  | 29                            |
| M/28     | ALL, 1st relapse       | III      | 6.33                   | 1.28                   | 10.00/0.100 | 23   | 28  | 18                            |
| F/38     | CML, chronic phase‡    | III      | 6.43                   | 2.75                   | 18.10/0.000 | 12   | 19  | 12                            |
| M/28     | CML, chronic phase     | III      | 8.48                   | 3.81                   | 3.60/0.000  | 14   | 22  | 10                            |
| F/6      | ALL, resistant in relapse‡ | III | 8.28  | 7.45                   | 8.70/0.000  | 13   | 20  | 8                             |
| M/23     | AML, 1st CR            | III      | 6.00                   | 3.10                   | 1.80/0.000  | 12   | 13  | 11                            |
| M/28     | AML, 3rd CR            | III      | 1.70                   | 1.20                   | 1.70/0.000  | 18   | 19  | 12                            |
| F/35     | AML, 1st CR            | III      | 2.00                   | 1.40                   | 1.40/0.000  | 11   | 15  | 16                            |

| CR | complete remission; IR | incomplete remission; RCR | resistant disease; ER | early remission. |

*Percentage of remissions with SBRTs before and after T cell depletion.
†Only 1,000 rad was administered, plus arabinoside ($k + [ARA]= k$ mg/kg).
‡[ARA]= $k +[ARA]= k$ mg/kg.
§Chemotherapy consisted of 22 g rad six times.
||Total lymphoid irradiation, 160 rad four times, was added.

Competent T lymphocytes from the marrow in vitro; (2) in vitro CFU-GM is not seriously affected by the procedures used; (3) rapid engraftment of all hematopoietic elements occurred in all patients; (4) None of the patients developed any clinical signs of acute or chronic GVHD with an observation period >16 months in the first patients; (5) the posttransplant course was generally smooth, with a short hospitalization period.

Of the protocol I patients, two are alive and well (six to 12 months), one died of rejection (on day 103), and two poor-risk patients relapsed (on days 48 and 111). Of the protocol II patients, two are alive and well >1 year (one with testicular relapse), two (one with chronic myelogenous leukemia [CML]) died of rejection (on days 52 and 76), and one poor-risk patient died of relapse (on day 76). Of the protocol III patients, six are alive and well (30 days to >1 year), two of three poor-risk patients received transplants during relapse died of relapse (on days 48 and 172), and the two CML patients died of rejection.

The single major problem that appears to be related to the T cell depletion procedure seems to be graft rejection occurring in five patients (1/6, protocol I; 2/5, protocol II; 2/10, protocol III), indicating that donor T cells play a role in abrogating residual host resistance. All attempts to retransplant rejected grafts even without cell depletion, with or without further conditioning, failed. It appears, therefore, that further conditioning pretransplant might prove useful in abrogating host resistance to T cell-depleted marrow allografts. Indeed, the last three patients who received transplants in protocol III received...
total lymphoid irradiation (150 rad four times in two days) immediately prior to the standard conditioning regimen.

The complete eradication of GVHD seems to be encouraging and may open new horizons for wider applications of BMT in clinical practice. However, further observations are necessary in controlled clinical trials in good-risk patients for careful evaluation of the overall effects of T cell depletion in long-term survival and incidence of leukemic relapses.

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hydroxyethyl starch. Alternatively, a mononuclear cell fraction was isolated by centrifugation over Ficoll-Hypaque.45 The cells were suspended in approximately 250 mL of balanced salt solution (containing Ca²⁺ or culture medium to give a cell concentration of no more than 10⁶/mL. Campath-1M was added to give a final concentration of 0.1 mg/mL and the suspension was mixed and left at room temperature for 10–15 min. Donor serum (or AB serum was added to a final concentration of 20–25% (v/v) and the suspension was incubated at 37 °C for 30–45 min. Usually the cells were then washed with fresh frozen plasma or saline containing human albumin with the intention of removing excess antibody and anaphylotoxins. Occasionally the whole mixture was used. T cell depletion was estimated either by E-rosetting or by immunophenotyping using suitable fluorescent-labelled mAb. In almost every case, the fraction of residual T cells was <1% of the final inoculum and in many cases residual T cells were undetectable. Experiments have shown that complement, although added at an optimal concentration, is likely to be the limiting factor46,47 and so it is likely that any residual antibody-coated cells which had not been lysed in vitro, would still be eliminated in vivo.

### Table II  Patient characteristics according to antibody protocol

<table>
<thead>
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<th>Transplants and follow-up</th>
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</table>

Adback of T cells

Because of concern about the risk of relapse and the belief that donor T cells contribute a beneficial anti-leukaemic effect, various methods have been used to add back small, controlled numbers of lymphocytes.12,15,46 In some cases, a fixed number has been added to the BM inoculum after treatment with Campath-1M and complement. In another scheme, used by the team at Jerusalem, the donor lymphocytes were given in graded doses to the recipient after the transplant.15 The rationale was to start with a very small, probably ineffective, dose and to give ten times more each fortnight until a maximum (of about 10⁷ per kg) was reached. Some patients with poor-risk leukaemia finally received donor T cells which had been activated with IL-2. If there were signs of GVHD at any stage, then no more donor lymphocytes were given. Most recently the timing of the first T cell adback has been delayed until about 2 months post-transplant and it has not been given at all if there were any sign of GVHD from the donor marrow. For the purposes of this analysis we have pooled all of the patients who actually received any form of T cell adback, irrespective of the precise dose or timing.

### Treatment of bone marrow with Campath-1G

Auffy coat or mononuclear cell fraction was prepared as before and Campath-1G was added to give a final concentration of 0.05–0.1 mg/mL. The mixture was incubated at room temperature for 30–45 min before infusion into the recipient without washing. In this case, it was anticipated that the excess antibody could also contribute to the immunosuppression of the host. It was not expected that T cells would be efficiently lysed in vitro under these circumstances as the IgG2b antibody is less lytic with complement than the IgM and no extra source of complement was added. The aim was to opsonize the donor T cells for clearance in the recipient. However, analysis by immunofluorescence in several patients has shown about 90% depletion of T cells during the in vitro incubation. This may be due to residual donor plasma in theuffy coat fraction as no T cell lysis was seen when mononuclear cells prepared on Ficoll-Hypaque were used (W. Fibbe & R. Willemze, University of Leiden, unpublished data); however, some cell-mediated killing in vitro may be possible.

### Statistical analysis

Acute GVHD was diagnosed and staged according to the Seattle criteria.48 Chronic GVHD was staged according to the clinical criteria prevailing at each centre; 'mild' and 'moderate' were both scored 'M' and 'extensive; or 'severe' were scored 'S'. Percentages of patients suffering graft failure or GVHD were calculated as a fraction of patients at risk from those complications. Patients were considered not evaluable for graft failure if they died before day 100 without evidence of engraftment, not evaluable for acute GVHD if they died before day 100 without acute GVHD and not evaluable for chronic GVHD if they died (or were last reported) before day 120 without
Control of graft-versus-host disease and graft rejection by T cell depletion of donor and recipient with Campath-1 antibodies. Results of matched sibling transplants for malignant diseases

G. Hale & H. Waldmann for Campath users

Department of Pathology, University of Cambridge, Cambridge, UK

Summary:

Campath-1 (CDw52) antibodies (IgM and IgG2b) have been used in vitro and in vivo for control of GVHD and prevention of rejection following bone marrow transplantation. Results of 951 patients with malignant disease transplanted from HLA-matched siblings are reported. Both Campath-1M and Campath-1G are shown to be effective when used in vitro for prevention of graft-versus-host disease (GVHD). Graft failure was reduced by addition of cyclosporin A (CsA) post-transplant and possibly also by total lymphoid irradiation (TLI) pre-transplant. However, treatment of the recipient with Campath-1G to deplete residual lymphocytes was more effective, reducing the incidence of graft failure from 21% to 9% (in the absence of CsA). GVHD was virtually eliminated and leukaemia-free survival was improved. However, the risk of relapse was increased by T cell depletion, certainly in CML and to a lesser extent in AML. Addition of donor T cells to the depleted bone marrow or early post-transplant restored the risks of GVHD, graft failure and relapse to much the same as without T cell depletion. One problem associated with the use of Campath-1G in vivo was a significant delay (by up to 7 days) in neutrophil engraftment. This was unlikely to be caused by toxicity to progenitor cells and we argue that small numbers of lymphocytes may be required to assist early engraftment, possibly by cytokine production. If this problem can be overcome, T cell depletion of donor and recipient may be a good alternative to conventional GVHD prophylaxis for matched sibling transplants, resulting in a superior quality of life for the survivors. It is also likely to be particularly beneficial in transplants for non-malignant diseases and transplants from unrelated donors.

Despite the undoubted advances in drug-based immunosuppression in recent years, GVHD is still a significant complication of BMT, particularly when the donor and the recipient are not HLA-matched siblings.

It is generally accepted that the most effective way of preventing both acute and chronic GVHD is by depletion of T lymphocytes from the donor BM. One of the simplest ways of achieving this was with the moAb Campath-1M which activates human complement and has been widely used by many transplant centres²-³ (Appendix, Protocol 01). However, the benefits of T cell depletion are largely negated by an increase in the risk of graft rejection and leukaemia relapse. These potential problems were noted in some of the early clinical studies²,4,6 and had been predicted from animal experiments.⁵-⁷ Subsequent analysis has shown a risk of graft failure of 10-20% in patients receiving T cell-depleted marrow from matched siblings although there has been some variation between different studies, reflecting differences in the degree of depletion and possibly also in the conditioning regimens.⁵,⁶,8-12 An increased risk of leukaemia relapse is most noticeable in patients with CML.¹³ The effect in acute leukaemias seems to be much smaller.¹⁴

Some groups have tried to overcome these problems of Protocol 01 by adding back small numbers of T cells to the BM inoculum. Although this did diminish graft rejection, GVHD was also restored to the about previous frequency and intensity.¹³,¹⁵,¹⁶ The effect of these efforts on relapse has been hard to assess because of the small numbers reported so far but in general it seems as though the status quo was restored. In other studies the problem of graft rejection has been tackled by the addition of other immunosuppressive agents such as CsA or total lymphoid irradiation (TLI)¹⁷ and they have met with some success, as will be shown here.

We have also investigated a new approach, based on experiments in animals which showed that treatment of the recipient with depleting anti-T cell antibodies could abolish rejection of T cell-depleted BM, even with reduced irradiation or when the donor and recipient differed across major histocompatibility barriers.⁹,¹⁸ Evidence from clinical studies suggests that graft failure is mainly due to rejection by host lymphocytes as donor-specific cytotoxic T cells have been isolated from several patients with graft rejection.¹⁹-²² Furthermore, in both monkeys and humans we observed that autologous BM depleted of T cells with Campath-1M engrafted normally whereas there was a high incidence of rejection of T cell-depleted BM from unrelated or HLA-mismatched donors.²³
Our aim was to obtain an antibody which could give lymphocyte depletion in the human comparable to that obtained in those experimental animals. Not all cell surface antigens are equally good targets for antibody attack but the Campath-1 (CDw52) antigen seemed to be a particularly good one.1.26,27 This abundant antigen is a small, lipid-anchored glycoprotein which is present on virtually all human lymphocytes. Its expression on B cells was not considered a problem in the context of BMT, rather it might be an advantage as depletion of B cells could reduce the potential target for EBV infection and lymphoproliferation during the period when there is little control by T cells. This has been a problem with some other methods of T cell depletion.26-29 Furthermore, if Campath-1 antibodies could be used to deplete lymphocytes in vivo, they might also contribute to the elimination of residual disease in patients with lymphoid malignancies.

The original IgM antibody Campath-1M, although extremely lytic with complement, gave only transient depletion of lymphocytes in patients with CLL or lymphoma.30 We therefore developed the rat IgG2b antibody Campath-1G, which has an almost identical specificity.31 The IgG2b subclass, besides being the best rat IgG for activating complement32 is also optimal for binding to human Fc receptors and activating cell-mediated killing.33 Campath-1G proved to be very efficient at depleting lymphocytes in vivo as was shown in several patients with lymphoid malignancies and in patients suffering steroid-resistant kidney graft rejection.34,35 In both studies, the treatment with Campath-1G gave a beneficial clinical response in many of the patients. Subsequently the humanized antibody, Campath-1H (IgG1), was constructed by genetic engineering using the complementarity-determining regions of Campath-1G.36 It is equally potent in vivo but is less immunogenic on repeated administration.37,38 However, studies in BMT using Campath-1G had already started before Campath-1H became available. We did not consider there was any advantage in using the humanized antibody in this context because the treatment was only short-term at a time when the level of immunosuppression is such that an antiglobulin response is unlikely.

The first objective was to see whether treatment with Campath-1G would decrease the risk of graft rejection in the context of 'standard' T cell depletion. This was not so easy to test because the actual incidence was comparatively low and so quite a large number of patients is needed to demonstrate an effect. For example, for a study to have a 90% chance of showing a significant reduction from 15% graft failure to 5%, then about 55 patients would be required. Initially we did not know whether the recipient's effector mechanisms would be compromised by the conditioning regimen, so it was possible that administration of antibody at the time of the transplant might not be effective. Therefore a study was designed where Campath-1G was given at 5-10 mg/day for 5 days before the start of chemoradiotherapy (Protocol 02).

Although this had the slight disadvantage that extra inpatient time was required, this protocol had several other advantages: (1) the degree of lymphopenia produced by Campath-1G could be measured, uncomplicated by other treatments, (2) adverse effects of the antibody could likewise be assessed, and (3) most of the antibody would be cleared by the time of the transplant and so be unlikely to contribute to extra depletion of the donor T cells. For this study, the T cell depletion with Campath-1M and other elements of the conditioning regimen were kept the same as in historical control groups. No other agents to prevent graft rejection (e.g. CsA, TLI) were given. The plan is to enrol 100 patients with acute leukaemia in first remission which will provide a homogenous group where the effect of the antibody protocol on relapse can also be observed. To date about 75 such patients have been enrolled by three transplant teams (Royal Free, London; University Hospital, Ulm; King Faisal Hospital, Riyadh). The interim results are very satisfactory but a detailed report will be published when the study is complete. Here we have included these study patients with a larger group of patients, including CML and advanced disease, who all received the same antibody protocol.

Meanwhile, other centres have explored the use of Campath-1G for prevention of GVHD, either adding it in vitro to the donor BM (Protocol 03),31,39,40 or by giving it in vivo at or around the time of transplant (Protocols 04 and 06).41 In both cases, it was intended that the antibody would also contribute to prevention of rejection. The logical development was to combine these treatments and give the antibody both in vivo and in vitro (Protocol 07). Although the numbers of patients treated with these newer protocols is still relatively small, it is clear that Campath-1G used in either way can reduce the incidence of GVHD.

In this report we focus on transplants from HLA-matched siblings for leukaemia and other malignant diseases. Similar protocols have been used in both unrelated donor transplant and in HLA-matched or mismatched transplants for non-malignant diseases. There have already been some reports of those studies.40,42 The results are broadly in agreement with those described here.

All the results described here are from open studies. There were several reasons why we did not consider it appropriate to carry out a prospective randomised trial. Few centres would be prepared to continue with the original T cell depletion protocol for a 'control' group, given the past experience of graft failure and the perception that the proposed new treatments would probably be of benefit. If the comparison were to be with 'standard' therapy (e.g. MTX/CsA), then we did not know what differences to expect in transplant-related mortality or leukaemia-free survival. There were too many possible variables in the antibody treatment to choose between (dose, timing etc) before a good trial could be designed. Therefore we have first explored these variables by testing alternative protocols and comparing the results with the most similar historical group.
Patients and methods

Transplants and conditioning regimens

A total of 986 transplants were reported from HLA-matched siblings for patients with leukaemia and other malignant diseases. Of these, 35 were excluded from analysis here either because antibodies other than Campath-1M or Campath-1G were used (2 cases) or because the available follow-up was < 100 days. The 951 transplants analysed were carried out at 30 centres between July 1983 and March 1993 (Appendix). During this time different protocols of antibody treatment were tested in roughly chronological order (Figure 1); however, several of them have been in use concurrently at different centres. Patients were transplanted for a typical range of diseases, 554 with acute leukaemia in CR1 or CML in CP1 and 397 with more advanced disease (Table I). The distribution among different antibody protocols was not uniform; for example, patients with CML were mostly treated according to the original protocol (01) (172 patients, median age 35 years) and only a small number (41 patients, median age 41 years) have been enrolled in subsequent protocols because of the feared increase in relapse after T cell depletion. These recent CML patients received additional anti-leukaemia conditioning with thiopeta, but it is too early to tell what impact that will have on relapse.

Conditioning regimens varied considerably. Seventytwo patients (8%) were conditioned with busulphan plus CY; the rest received CY/TBI with or without other anti-leukaemia drugs. The schedule for TBI varied according to the usual practice and facilities available at each centre. Some patients also received TLI in order to reduce the risk of graft rejection. No post-transplant immunosuppression was given in the majority of cases (60%, 64%). Two patients received MTX and 6 received MTX/CsA. They are included with the 334 who received CsA post-transplant. A summary of the patient characteristics and conditioning according to the different antibody protocols is given in Table II.

Monoclonal antibodies

Campath-1M (IgM) and Campath-1G (IgG2b) are rat mAb which recognise the CDw52 antigen. Initially they were prepared in the Department of Pathology from ascitic fluid of rats inoculated with hybrid myeloma cells. The antibodies were purified by precipitation with ammonium sulphate, dialysed against phosphate-buffered saline and sterilised by membrane filtration. Subsequently, they were prepared from the culture supernatant of cells grown in hollow-fibre fermentors (Acusyst Jr, Endotronics). Since 1990, this has been carried out in the Therapeutic Antibody Centre, Regional Transfusion Centre, Cambridge. Campath-1M was purified by precipitation with ammonium sulphate as before. Campath-1G was purified by affinity chromatography on Protein A, followed by ion exchange on S-Sepharose under the control of a Biopilot chromatography system (Pharmacia). The cell lines were tested for microorganisms and viruses according to EC guidelines and the purified antibodies were submitted to a panel of tests to ensure their biological activity and freedom from contaminants.

Treatment of BM with Campath-1M and human complement

Marrow was aspirated and a buffy coat fraction was prepared by centrifugation or by sedimentation with

| Table I Distribution of patients by disease and status at transplant |
|---------------------------------|-----------------|
| Disease                        | No. of patients |
| ALL-CR1                        | 98              |
| ALL-CR2                        | 84              |
| ALL-others                     | 63              |
| AML-CR1                        | 235             |
| AML-CR2                        | 31              |
| AML-others                     | 55              |
| CML-CP1                        | 221             |
| CML-others                     | 78              |
| Myeloma                        | 29              |
| Other malignancies             | 57              |
| Total                          | 951             |

Figure 1 Protocols of antibody treatment for prevention of GVHD and graft rejection. The approximate timing of in vivo antibody treatment with Campath-1G is shown. The dose was usually 5-10 mg/day. Protocol 05 was not used for HLA-matched siblings.
hydroxyethyl starch. Alternatively, a mononuclear cell fraction was isolated by centrifugation over Ficoll-Hypaque. The cells were suspended in approximately 250 ml of balanced salt solution (containing Ca²⁺ or culture medium to give a cell concentration of no more than 10⁹/ml. Campath-1M was added to give a final concentration of 0.1 mg/ml and the suspension was mixed and left at room temperature for 10-15 min. Donor serum (or AB serum was added to a final concentration of 20-25% (v/v) and the suspension was incubated at 37°C for 30-45 min. Usually the cells were then washed with fresh frozen plasma or saline containing human albumin with the intention of removing excess antibody and anaphylatoxins. Occasionally the whole mixture was infused. T cell depletion was estimated either by E-rosetting or by immunophenotyping using suitable fluorescent-labelled mAb. In almost every case, the fraction of residual T cells was <1% of the final inoculum and in many cases residual T cells were undetectable. Experiments have shown that complement, although added at an optimal concentration, is likely to be the limiting factor⁴⁸,⁴⁹ and so it is likely that any residual antibody-coated cells which had not been lysed \textit{in vitro}, would still be eliminated \textit{in vivo}.

\textbf{Treatment of bone marrow with Campath-1G}

A buffy coat or mononuclear cell fraction was prepared as before and Campath-1G was added to give a final concentration of 0.05-0.1 mg/ml. The mixture was incubated at room temperature for 30-45 min before infusion into the recipient without washing. In this case, it was anticipated that the excess antibody could also contribute to the immunosuppression of the host. It was not expected that T cells would be efficiently lysed \textit{in vitro} under these circumstances as the IgG2b antibody is less lytic with complement than the IgM and no extra source of complement was added. The aim was to opsonize the donor T cells for clearance in the recipient. However, analysis by immunofluorescence in several patients has shown about 90% depletion of T cells during the \textit{in vitro} incubation. This may be due to residual donor plasma in the buffy coat fraction as no T cell lysis was seen when mononuclear cells prepared on Ficoll-Hypaque were used (W. Fibbe & R. Willemze, University of Leiden, unpublished data); however, some cell-mediated killing \textit{in vitro} may be possible.

\textbf{Addback of T cells}

Because of concern about the risk of relapse and the belief that donor T cells contribute a beneficial anti-leukaemic effect, various methods have been used to add back small, controlled numbers of lymphocytes. In some cases, a fixed number has been added to the BM inoculum after treatment with Campath-1M and complement. In another scheme, used by the team at Jerusalem, the donor lymphocytes were given in graded doses to the recipient after the transplant. The rationale was to start with a very small, probably ineffective, dose and to give ten times more each fortnight until a maximum (of about 10⁹ per kg) was reached. Some patients with poor-risk leukaemia finally received donor T cells which had been activated with IL-2. If there were signs of GVHD at any stage, then no more donor lymphocytes were given. Most recently the timing of the first T cell addback has been delayed until about 2 months post-transplant and it has not been given at all if there were any sign of GVHD from the donor marrow. For the purposes of this analysis we have pooled all of the patients who actually received any form of T cell addback, irrespective of the precise dose or timing.

\textbf{Statistical analysis}

Acute GVHD was diagnosed and staged according to the Seattle criteria.⁵⁰ Chronic GVHD was staged according to the clinical criteria prevailing at each centre; 'mild' and 'moderate' were both scored 'M' and 'extensive, or 'severe' were scored 'S'. Percentages of patients suffering graft failure or GVHD were calculated as a fraction of patients at risk from those complications. Patients were considered not evaluable for graft failure if they died before day 20 without evidence of engraftment, not evaluable for acute GVHD if they died before day 100 without acute GVHD and not evaluable for chronic GVHD if they died (or were last reported) before day 120 without
chronic GVHD. Patients who suffered graft failure were subsequently not considered to be evaluable for GVHD (even if they did suffer GVHD from an unpurged 'top-up' or second transplant). This method of calculation is likely to slightly over-estimate the incidence of these complications compared with the true actuarial incidences but information about the date of onset of graft failure or GVHD was not always available. Because the aim of these studies was to simultaneously reduce the risk of either complication, a composite result was also calculated. A 'bad' outcome was defined as graft failure (complete or partial) or severe (grade III or IV) acute GVHD or severe chronic GVHD. Relapse in CML was defined by haematological criteria and in this analysis we did not consider cases of relapse which were detected only cytogenetically or by PCR.

Actuarial survival data reported in the text are at 2 years unless otherwise stated, with standard errors calculated as described by Peto et al. Transplant-related mortality was defined as death from any cause except following haematological relapse. If a patient suffered relapse, their contribution to the actuarial plot was censored from that date. For univariate analyses the chi-square test (GVHD and graft failure), the Mann–Whitney U-test (time to engraft) or the log rank test (survival and relapse) were used as appropriate. Comparisons using the log-rank test were evaluated from 0–2000 days. Probability (p) values were one-sided as we could predict the sign of the differences in advance.

The database contained information on centre, sex, age, disease, status at transplantation, TBI dose rate, total dose, number of fractions and timing, TLI dose, chemotherapy, antibodies in vivo, donor sex, date of transplant, cell dose, T cell dose, method of T cell depletion in vitro. T cell addback, post-transplant immunosuppression, time to reach neutrophils 0.5 × 10⁹/l, graft failure, acute and chronic GVHD, time to relapse, survival and cause of death. Follow-up was requested for all patients between April and June 1993. The response was good and in fewer than 5% of the patients was no reply obtained from a centre or the patient was reported as 'lost to follow-up' (usually because of returning to a foreign country).

### Results

**T cell depletion in vitro with Campath-1M and complement (Protocol 01)**

The impact of T cell depletion with Campath-1M on GVHD and graft failure has been reported before. With more patients and longer follow-up (median 7.3 years) we are now in a position to assess the effects of CsA and TLI and to evaluate the risk of leukaemia relapse (Table III). The overall incidence of graft failure (early or late, partial or complete) was 18%. Patients who received post-transplant immunosuppression with CsA had less graft failure (13%) than those who did not (21%) (p < 0.04). Likewise, the rate of graft failure was lower in patients who received TLI (12%) than in those who did not (19%) but this did not reach statistical significance. However, when we looked at the overall rate of severe complications (graft failure or severe GVHD), TLI showed a significant advantage (p < 0.02). A small proportion of patients received both CsA and TLI which confounded the individual effects. We therefore looked separately at the effects of CsA in patients who did or did not receive TLI (Table III). Both TLI and CsA seemed to give a similar reduction in graft failure. There was also slightly less GVHD in the patients who received TLI. Overall, both treatments independently reduced the combined incidence of these complications.

It was only in the groups receiving additional CsA that we could see a significant reduction in transplant-related mortality (45% without CsA, 28% with CsA, p < 0.0001). This was reflected in superior survival of the CsA group (39% without CsA, 54% with CsA, p < 0.006) but because of a slightly higher risk of relapse, the difference in leukaemia-free survival was only marginal (35% without CsA, 42% with CsA). It is not clear why the short-term advantage seen with TLI was not reflected in a reduced transplant-related mortality because the two groups of patients were comparable for all the other prognostic factors which had been reported (Table II).

Although many of the improvements seen with these immunosuppressive treatments were modest, it gave some encouragement to the idea that graft failure could

<table>
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<th>Table III</th>
<th>Graft failure and GVHD in original protocol: effect of CsA and TLI</th>
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<tr>
<td>01</td>
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<td>CsA</td>
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Results are compared with the first group (no TLI or CsA). Those which are significantly better are highlighted in bold script, together with the p value.

*Either includes patients who suffered graft failure and/or severe GVHD*
be overcome and we hoped that treatment with the moAb would have a more decisive impact.

The risk of relapse for patients with early leukaemia transplanted according to Protocol 01 is shown in Figure 2. With up to 10 years follow-up, the risk of relapse in ALL and AML shows a plateau at approximately 30%, with most cases occurring in the first year. In CML, the risk of haematological relapse continues to rise for at least 5 years, reaching an apparent plateau of about 65% by 7 years.

**Campath-1G for control of rejection and GVHD (Protocols 02–07)**

The results to date using Campath-1G in vivo and/or in vitro in order to reduce both graft rejection and GVHD are shown in Table IV and Figure 3. They are compared with the results obtained using the original protocol. In all of the categories there were some patients who received post-transplant immunosuppression with CsA, although this was less frequent with the more recent protocols than originally. As we have shown above, CsA seems to reduce the risk of graft failure with T cell depletion. So that the data are more comparable, we have only shown the results in the patients who received no post-transplant immunosuppression. The results and conclusions were the same when the CsA-treated patients were included but in the absence of CsA we can much more confidently infer an anti-GVHD effect of the antibody treatments.

Protocol 02 shows a significantly lower incidence of both graft failure and acute GVHD. This resulted in a substantially lower transplant-related mortality compared with protocol 01 (21% vs 45%, p < 0.0001) with significantly improved survival and leukaemia-free survival (48% vs 35%, p < 0.004). The results on this protocol were slightly superior to the use of CsA with T cell depletion but not significantly so. The overall risk of relapse is almost identical between Protocols 01 and 02 but it would be premature to draw any firm conclusions because of the comparatively short follow-up of Protocol 02 and the difference in the mix of patients (e.g. fewer patients with CML in protocol 02).

In Protocol 03 (Campath-1G in vitro) the incidence of either GVHD or graft failure is very low. It provides very clear evidence that GVHD can be abrogated using Campath-1G without the need for exogenous complement. The number of patients is too small for the reduced incidence of graft failure to be statistically significant. However, the transplant-related mortality is lower than for the comparable original group (20% vs 45% p < 0.04) and the leukaemia-free survival is better (68% vs 35%, p < 0.01). It should be noted that all the patients in this group received TLI which may have helped to reduce the rate of graft failure. Nevertheless, the improvement in transplant-related mortality and leukaemia-free survival is still maintained when the group is compared with only those patients from the original protocol who also received TLI.

The combined results from Protocols 04 and 06 (Campath-1G in vivo only) show a low incidence of graft failure and acute GVHD but a substantially increased incidence of chronic GVHD. Most of the cases of severe chronic GVHD occurred on Protocol 04 where the Campath-1G was stopped on the day of transplant. These results provide clear evidence that Campath-1G in vivo can deplete the donor T cells sufficiently to prevent much of the acute GVHD that would otherwise have been expected but presumably a few T cells survive and give rise to chronic GVHD in many patients.

On the basis of the results so far, the combination of Campath-1G in vivo and in vitro (Protocol 07) was expected to give optimal control of both GVHD and graft failure. Control of GVHD was indeed impressive with no cases of acute GVHD > grade I or chronic

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**Figure 2** Actuarial risk of leukaemia relapse in patients receiving BM depleted of T cells with Campath-1M (Protocol 01). (a) ALL-CR1; (b) AML-CR1; (c) CML-CPI. The shaded areas indicate the standard errors of the actuarial estimates.
Table IV  Graft failure and GVHD according to antibody protocol (no post-transplant immunosuppression)

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<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.00110</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0068</td>
</tr>
<tr>
<td>07</td>
<td>CP1G</td>
<td>CP1G</td>
<td>21</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

Results are compared with the first group (Protocol 01). Those which are significantly better are highlighted in bold script, those which are worse are in italic. In each case the p value is also given.

Although the data presented in this table are of only patients who received no post-transplant immunosuppression, essentially identical results were obtained when all patients were analysed irrespective of post-transplant immunosuppression.

GVHD of any grade being reported to date. However, there have been a total of seven cases of graft failure in 28 patients (including two who received CsA). Perhaps the explanation for this can be found in the rates of engraftment which are described below.

**T cell addback**

Several protocols for adding back T cells were used. In 118 cases the donor BM was first depleted with Campath-1M and complement and in 70 cases Campath-1G was used. None of the patients received additional Campath-1G in vivo. The results from the different methods were essentially indistinguishable and so they have been pooled (Table V). About half of the patients received post-transplant CsA and these have been analysed separately because of the effect which CsA had in the original protocol. Whether or not CsA was given, T cell addback gave a significant reduction in graft failure and a significant increase in GVHD, both acute and chronic. Essentially the status quo was restored, although in the case of patients who did not receive CsA, the amount of GVHD was probably not as great as would have been seen with no manipulation of the donor T cells. With CsA, the transplant-related mortality was significantly worse with T cell addback compared with the original protocol (42% vs 28%, p < 0.005) but there was no difference without CsA because of the poorer results in the original protocol (44% vs 45%).

Overall (with or without CsA), the risk of relapse after T cell addback was significantly less than with the original protocol (26% vs 38%, p < 0.007). Because of the increase in transplant-related mortality, the leukaemia-free survival was little different (42% vs 38%). However, the patients who received T cell addback included significantly more with advanced disease (55% vs 39%, p < 0.0001) so the strategy may have been more successful than is immediately apparent. When we analysed all patients with early disease (CR1 or CP1) then T cell addback still reduced the risk of relapse compared with T cell depletion by any method (16% vs 27%, p < 0.02). Leukaemia-free survival was still the same (49% vs 50%). When we analysed relapse and leukaemia-free survival separately according to the disease and status at the time of transplant, there were no significant differences but this is probably because the numbers of patients in each category are too small.

**Rate of engraftment**

Despite the problem of graft rejection in a minority of patients, prompt neutrophil engraftment is the norm following T cell depletion of the donor bone marrow. We analysed the effect of T cell depletion in vitro and in vivo on engraftment rate in this group of patients and compared them with patients who received similar protocols of antibody treatment for transplants from unrelated or mismatched donors (Table VI).

Using the original protocol (01) the median time to reach neutrophils 500 × 10⁹/l (for those patients who did engraft) was 19–20 days. Treatment with CsA or TLI made no difference. Likewise, using Campath-1G in vitro (Protocol 03), rapid engraftment was seen (median 16–17 days). When T cells were added back, using either protocol, engraftment was still rapid (17–18 days) although in many cases the T cells were not given until after engraftment was documented. However, in every protocol where Campath-1G was given in vivo, there was a significant delay in neutrophil engraftment ranging from 23 to 27 days. The overall median for all evaluable patients who received antibody in vivo was 24 days whereas the median for patients who only received antibody in vitro was 19 days (p < 0.001).

It is apparent that the same delay in engraftment is not seen in all of the corresponding groups of unrelated/mismatched transplants. This is particularly so for Protocol 02 where the mismatched/unrelated transplants engraft as promptly as the original T-depleted group (note that patients who failed to engraft were not included).

The timing of the in vivo antibody seems to be important and the more that was given close to the transplant, the greater is the delay in engraftment. The original intention in Protocol 02 had been to give the Campath-1G well before the transplant and this was
always done with the unrelated/mismatched transplants. However, it happened that one centre often gave the 5-day course of Campath-1G much closer to the transplant for matched siblings, the final day usually being day -1. Forty-one evaluable transplants performed at this one centre showed an extra delay in engraftment (median day 28) compared with the 87 transplants performed at all other centres (median day
Table V  Graft failure and GVHD in patients who received T cell addback

<table>
<thead>
<tr>
<th>Protocol</th>
<th>No. of patients</th>
<th>Graft failure</th>
<th>Percentages</th>
<th>Either*</th>
<th>Actuarial at 2 years ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acute GVHD</td>
<td>Chronic GVHD</td>
<td></td>
<td>Transplant mortality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3/4</td>
<td>M</td>
<td>S</td>
</tr>
<tr>
<td>01</td>
<td>+ T cells</td>
<td>None</td>
<td>34</td>
<td>0.0068</td>
<td>6</td>
</tr>
<tr>
<td>03</td>
<td>+ T cells</td>
<td>None</td>
<td>55</td>
<td>0.0015</td>
<td>25</td>
</tr>
<tr>
<td>01</td>
<td>+ T cells</td>
<td>CsA</td>
<td>84</td>
<td>0.024</td>
<td>15</td>
</tr>
<tr>
<td>03</td>
<td>+ T cells</td>
<td>CsA</td>
<td>15</td>
<td>0.00122</td>
<td>22</td>
</tr>
</tbody>
</table>

Results are compared with the first group in Table IV (Protocol 01)
Significantly different results are highlighted as before.

Table VI  Engraftment rate: comparison of matched siblings with mismatched and unrelated donors for different antibody protocols

<table>
<thead>
<tr>
<th>Protocol No.</th>
<th>In vivo</th>
<th>In vitro</th>
<th>Matched siblings</th>
<th>Mismatched/unrelated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. evaluable</td>
<td>Median day to neutrophils</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 x 10⁹/l</td>
</tr>
<tr>
<td>01</td>
<td>None</td>
<td>CP1M</td>
<td>429</td>
<td>19</td>
</tr>
<tr>
<td>02</td>
<td>None</td>
<td>CP1M</td>
<td>107</td>
<td>18</td>
</tr>
<tr>
<td>03</td>
<td>None</td>
<td>CP1M</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>04/6</td>
<td>None</td>
<td>CP1G</td>
<td>68</td>
<td>17</td>
</tr>
<tr>
<td>07</td>
<td>CP1G</td>
<td>CP1G</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01</td>
<td>None</td>
<td>CPIM</td>
<td>429</td>
<td>19</td>
</tr>
<tr>
<td>02</td>
<td>None</td>
<td>CPIM</td>
<td>107</td>
<td>18</td>
</tr>
<tr>
<td>03</td>
<td>None</td>
<td>CPIM</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>04/6</td>
<td>None</td>
<td>CP1M</td>
<td>68</td>
<td>17</td>
</tr>
<tr>
<td>07</td>
<td>CP1G</td>
<td>CP1G</td>
<td>37</td>
<td>19</td>
</tr>
</tbody>
</table>

Results are pooled irrespective of post-transplant immunosuppression or TLI (which made no difference to the engraftment rates)
Patients who failed to engraft are excluded
The median day to engraft for each group is compared with that for the 429 patients treated according to Protocol 01

![Neutrophil engraftment graph](image)

Figure 4  Time to neutrophil engraftment: comparison of in vitro with in vivo T cell depletion. The results are pooled from all patients receiving in vitro depletion alone (Protocols 01 and 03) or in vivo depletion (Protocols 02, 04, 06, 07). Patients who failed to engraft or who received T cell addback are excluded.

24. \( p < 0.001 \). Nevertheless, the 87 transplants were still delayed compared with the original protocol \( (p < 0.005) \).

Immune reconstitution and infections post-transplant

We did not centrally collect data on the recovery of lymphocyte subsets or the incidence of non-fatal infections because of the huge amount of complex reporting and analysis this would have entailed. We can say that lymphoproliferative disease was rare. Only five cases were reported which is equivalent to an actuarial risk of approximately 1% at 5 years. The number of fatal infections late after transplant certainly did not seem to be significantly more than that seen with conventional GVHD prophylaxis, because transplant-related mortality did not increase much after the first 9 months (Figure 3). However, individual centres have observed relatively slow lymphocyte regeneration following T cell depletion with Campath-IM²¹,²² and some have found a high incidence of CMV viremia (A. Bacigalupo (Genoa) & D. Bunjes (ULm), unpublished data).

Comparison of transplant outcome with published results using MTX/CsA

The generally accepted 'standard' prophylaxis for GVHD is currently the combination of a short course of MTX with CsA.³³,³⁴ Results for 580 patients with early disease have been published by the International Bone Marrow Transplant Registry (IBMTR).³⁵ We compared the pooled results for 466 patients with early disease who received T cell depletion (by any protocol) and no T cell addback (Table VII). As expected, the incidence of both acute and chronic GVHD was
Table VII  Outcome for patients with early disease compared with published data using MTX/CsA (at 2 years)

<table>
<thead>
<tr>
<th></th>
<th>ALL + AML + CML</th>
<th>ALL- CRI</th>
<th>AML- CRI</th>
<th>CML- CRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campath</td>
<td>IBMTR</td>
<td>Campath</td>
<td>IBMTR</td>
<td>Campath</td>
</tr>
<tr>
<td>No. of patients</td>
<td>460</td>
<td>84</td>
<td>196</td>
<td>192</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>14 ± 3</td>
<td>18 ± 9</td>
<td>27 ± 7</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>20 ± 4</td>
<td>25 ± 12</td>
<td>40 ± 9</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>TRM (at 2 years)</td>
<td>32 ± 6</td>
<td>28 ± 10</td>
<td>33 ± 10</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>Relapse (at 2 years)</td>
<td>27 ± 0</td>
<td>27 ± 14</td>
<td>27 ± 10</td>
<td>4 ± 7</td>
</tr>
<tr>
<td>LFS (at 2 years)</td>
<td>50 ± 6</td>
<td>52 ± 12</td>
<td>59 ± 7</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>DFS (approximately)</td>
<td>40</td>
<td>39</td>
<td>44</td>
<td>35</td>
</tr>
</tbody>
</table>

Results for all patients with early disease were pooled, irrespective of antibody protocol. Patients who received T cell ablation were excluded. They are compared with published data for a similar cohort of patients who received conventional GVHD prophylaxis. In this table (and in Table VIII), unlike the others, the results are presented ± 95% confidence interval, which for the Campath group was taken to be approx × the SE (which is quoted elsewhere).

substantially lower with T cell depletion. The transplant-related mortality was not improved, presumably because of the complications associated with graft failure. An increase in the risk of relapse with T cell depletion is striking in the case of CML and modest in the case of AML. For ALL, there does not seem to be a significant increase in relapse risk. Overall the leukaemia-free survival is lower for the T cell-depleted patients, most notably those with CML.

It is very difficult to measure the effect of chronic GVHD on the quality of life of the long-term survivors who received conventional GVHD prophylaxis, although it is accepted that this is still a significant problem for many patients. We made an approximate calculation of ‘disease-free’ survival (meaning free from leukaemia or GVHD) by multiplying the leukaemia-free survival by the fraction of patients who were free from chronic GVHD. This is not strictly correct because some of the patients with severe chronic GVHD will have died as a result and those with milder forms are likely to be at lesser risk of relapse and so more likely to survive. However, the two errors are opposite and will tend to cancel out. By this criterion, the patients who received T cell-depleted transplants tend to do slightly better.

The results are also aligned with an IBMTR report for patients who received T cell-depleted BM in order to compare the risks of relapse (Table VIII). The IBMTR data were calculated at 3 years and patients who failed to engraft were excluded, so we did the same. In practice it made very little difference to the result. The IBMTR data will include many different methods of T cell depletion and there is likely to be some overlap of patients with our database. The results show a smaller risk of relapse in each category for patients in the Campath-1 users database but the differences are unlikely to be statistically significant.

Discussion

We have described here one of the largest collections of data about T cell depletion in allogeneic BMT and from it we can draw some important conclusions.

Both Campath-1G and Campath-1M are equally effective in vitro for prevention of both acute and chronic GVHD. Technically, they are simple to use and have been employed with equal success by large and small transplant centres. Previous studies with other opsonizing antibodies were quite unsuccessful, which emphasizes the point that the antibody isotype and/or target antigen are critically important for cell depletion.

We have also shown that administration of Campath-1G in vivo is an alternative way of preventing acute GVHD, although the incidence of chronic GVHD is then greater, presumably because the effective antibody dose contacting the donor T cells is very much smaller, pabody < 5% of the concentration achieved in vitro. The original IgM antibody relied on complement for T cell depletion. We cannot assess the extent to which complement-dependent lysis or ADCC is important for the action of Campath-1G; however, our previous studies using a non-Fc binding lgG in vivo led us to believe that cell-mediated killing is important for its action. If so, the present results imply that the requisite effectors are still sufficiently active after intensive chemoradiotherapy.

The use of Campath-1G in vivo before conditioning with CY/TBI (Protocol 02) allowed us to extend the observations on the extent of lymphopenia and the side-effects it causes. These studies (D. Bunjes, G. Prentice & D. Spence, unpublished data) confirm the original findings already reported for Campath-1G and Campath-1H in BMTs, kidney transplants and rheumatoid arthritis. In brief, the first dose of antibody causes a substantial lymphopenia which is accompanied by the cytokine release effect typical of many lymphocyte-directed antibodies. Subsequent doses have lesser side-effects but tend to increase the degree of lymphopenia.

Our principal objective in using Campath-1G was to reduce the risk of transplant rejection by eliminating the residual host T cells. Sensitive limiting dilution assays showed that the extent of in vivo depletion by Campath-1G is comparable or greater than that achieved with busulphan/CY or TBI. Some centres also studied the use of conventional immunosuppressive agents, CsA or TLI, to achieve the same end. The
results suggest that CsA or TLI reduce the risk of graft failure by about one-third (from 23% to about 15%) whereas Campath-1G in vivo reduces it slightly more (to 9%) (Tables III and IV). The lowest rate of combined complications (graft failure and/or GVHD) was obtained with the combination of Campath-1G plus Campath-1M (Table IV). Only small numbers of patients have been given combined treatment (e.g. TLI plus CsA (Table III) or Campath-1G plus CsA, data not shown) but on the whole they have done very well. However, it is extremely difficult to assess the activity of each agent in a study where they were combined, even if ultimately this might be the best treatment. This is exemplified in Protocol 03 where patients were given TLI with Campath-1G in vitro. The incidence of graft failure (and GVHD) was very low but we cannot tell whether this is mainly due to the effect of the TLI or whether the excess Campath-1G infused with the BM also helped to deplete host T cells.

Particular teams have used moAb to prevent graft rejection, particularly the collaborative European Immunodeficiency Group who have used a combination of non-depleting CD2 and CD11a (LFA-1) antibodies, which can block the activity of T cells and are tolerogenic in some animal models. It is difficult to compare our results because the circumstances are very different. On the basis of animal experiments, we expect that a combination of cell-depleting and function-blocking antibodies would be likely to create the best situation for donor-specific tolerance but it is clear that the individual roles of the components of such a mixture needs to be clinically evaluated first.

One of the unexpected effects of in vivo therapy with Campath-1G was a significant delay of about a week in neutrophil engraftment. This was not seen in protocols which involved only in vitro treatment with Campath-1M or Campath-1G. We therefore think that it is very unlikely that the antibody is exerting an effect directly on the BM progenitors, e.g. direct toxicity or inhibition of homing or proliferation, as the patients who received marrow treated in vitro would presumably suffer the worst. Furthermore, recent experiments with Campath-1H show no evidence for Campath-1 expression on long-term colony-forming cells. It is conceivable that Campath-1G has a damaging effect on the stem cell supporting microenvironment, i.e. the stromal cells, but if that were the case we would expect an effect of at least equal magnitude in the patients who received mismatched or unrelated BM. In fact, paradoxically, they tend to engraft more rapidly (Table VI). A hypothesis which might explain all the observations is that lymphocytes (we cannot say whether T cells or B cells or both) are able to secrete cytokines which directly or indirectly stimulate the growth and differentiation of the progenitors during the critical few weeks after the transplant. We would propose that only very small numbers of lymphocytes are needed. Donor or host cells would suffice. It would be only when both are depleted below a small critical threshold that delayed engraftment would be observed. In transplants from less well matched donors there would be a greater scope for immediate lymphocyte proliferation due to alloreactivity. This in itself might generate the required cytokines, or at least expand the very small pool which remain after T cell depletion of donor and recipient.

It is well documented that T cell depletion results in a significant increase in the risk of relapse following BMT from HLA-matched siblings for CML. The effect is much greater than would be expected merely from the abrogation of GVHD and this is taken as evidence that the GVL and GVH effects of donor T cells are not identical. Surprisingly, there seems to be much less relapse in patients receiving T cell-depleted transplants from unrelated donors. We still wonder whether the absence of T cells allows a preferential expansion of the malignant clone during the early post-transplant recovery, although early evidence that relapse was associated with delayed engraftment has not been substantiated in the present analysis. In due course this hypothesis might be tested by analysis of the T cell addback data according to the time of re-infusion: currently the follow-up on those patients is too short.

In the case of acute leukaemia, it is less clear whether T cell depletion has a detrimental effect on relapse. The few prospective randomised trials carried out so far were too small to detect an effect. Analysis of pooled data by the IBMTR suggests that the effect is significant in AML but only marginal in ALL. Here we have a rather larger group of patients (probably including some overlap with the IBMTR) and obtain very similar results although the risk of relapse is slightly lower in each category (Table VIII). Comparison with the registry results for transplants using MTX/CsA shows a very similar risk of relapse in ALL-CRI, slightly higher in AML-CRI and substantially higher in CML-CP1 (Table VII). In this comparison, T cell depletion is unfavourable unless the morbidity associated with chronic GVHD is taken into account. However, the T cell depletion results were pooled from all the protocols described here, including those which had relatively poor transplant-related mortality due to graft failure (Protocols 01 and 07) or GVHD (Protocols 04 and 06). Currently the transplant-related mortality in Protocols 02 and 03 is lower than reported for MTX/CsA; we do not yet have sufficient numbers or follow-up to comment on relapse, but leukaemia-free survival so far is satisfactory (Table IV, Figure 3).

There have been several attempts to separate the beneficial and harmful effects of donor T cells. Based on data which suggest that GVL and GVH effects may be distinct. In some cases depletion of just one T cell subset has been tried. However, the results have not been better than with complete T cell depletion. Another approach, which has been tried here, was to deplete the marrow as effectively as possible then add back a small defined number of donor T cells. The problem is that it is impossible to define in advance the ideal number which will not cause GVHD as this will be a threshold phenomenon where the level depends on the particular genetic make up of donor and recipient, among other factors. One way to
Table VIII  Outcome for patients with early disease compared with published data using T cell depletion (at 3 years)

<table>
<thead>
<tr>
<th></th>
<th>ALL + AML + CML</th>
<th>ALL-CRI</th>
<th>AML-CRI</th>
<th>CML-CRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Campath IBMTR</td>
<td>Campath IBMTR</td>
<td>Campath IBMTR</td>
<td>Campath IBMTR</td>
</tr>
<tr>
<td>No. of patients</td>
<td>401</td>
<td>21 ± 4</td>
<td>84</td>
<td>170</td>
</tr>
<tr>
<td>Acute GVHD</td>
<td>14 ± 3</td>
<td>18 ± 9</td>
<td>26 ± 10</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>20 ± 4</td>
<td>25 ± 10</td>
<td>34 ± 13</td>
<td>54 ± 12</td>
</tr>
<tr>
<td>Relapse (at 3 years)</td>
<td>35 ± 6</td>
<td>401</td>
<td>69</td>
<td>84</td>
</tr>
</tbody>
</table>

Results are calculated as in Table VI and compared with published data for T-depleted transplants. In both cases, patients with graft failure were excluded (most of them would have died before being at risk of relapse but a few who received autologous BM may have relapsed). Inclusion of these patients in the Campath group made essentially no difference to the results.

minimise this problem is to give the T cells in graded doses over several weeks, stopping if GVHD occurs. However, it is still hard to control the extent of GVHD and the overall incidence is similar to that seen without T cell depletion. We have been able to show that addback of donor T cells results in a decrease in the risk of relapse, although the numbers treated so far are relatively small, so the effect was only significant when results from patients with different diseases are pooled. Nevertheless, separation of GVH from GVL is not yet demonstrable.

At present the tetrad of immunological problems associated with BMT – GVHD, rejection, relapse, infection – still remain interconnected. Measures which decrease one are likely to increase the others. We believe the long-term aim should be to isolate these problems and deal with them individually. A possible way forward is suggested by the recent advances in semi-quantitative analysis of minimal residual disease and the demonstrations that donor T cell infusions can be used to effectively treat outright relapse in some cases. We wonder whether T cell depletion of donor and recipient might be used to ensure a good recovery from the transplant procedure without GVHD or rejection and then donor T cells only be added later if there is evidence that a definite tumour burden still remains. Advances in the understanding of cytokines and their clinical application might lead to further improvements in the management of the post-transplant haemopoietic and immune reconstitution.

Of course, the problem of increased relapse is not relevant in transplants for non-malignant conditions such as aplastic anaemia and inherited disorders of the blood system. That would be an ideal situation to use donor and recipient T cell depletion for control of GVHD and rejection. So far relatively few patients have been treated but the results are very encouraging. We also expect that T cell depletion will have an important role in unrelated donor transplants where the immunological complications are relatively more severe.

Acknowledgements

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71. SLAVIN S, ACKERSTEIN A, WEISS L, NAGLER A, OR R, NAPARSTEK E. Immunotherapy of minimal


Appendix

Participating transplant centres (number of patients in parentheses): Queen Elizabeth Medical Centre, Birmingham, UK (11); Institute of Haematology Seragnoli, Bologna, Italy (24); Royal Hospital for Sick Children, Bristol, UK (20); Jules Bordet Hospital, Brussels, Belgium (6); Addenbrooke’s Hospital, Cambridge, UK (11); Groote Schuur Hospital, Cape Town, South Africa (30); University of Wales Medical School, Cardiff, UK (22); Hospital Cantonal, Geneva, Switzerland (57); San Martino Hospital, Genoa, Italy (42); Hadassah University Hospital, Jerusalem, Israel (187); Royal Free Hospital, London, UK (79); St. George’s Hospital, London, UK (1); Hammersmith Hospital, London, UK (77); Institute of Child Health, London, UK (12); The London Clinic, London, UK (26); Royal Marsden Hospital, London, UK (10); University College Hospital, London, UK (5); Westminster Hospital, London, UK (47); St. James University Hospital, Leeds, UK (31); Royal Infirmary, Leicester, UK (5); University Medical Center, Leiden, The Netherlands (39); Royal Infirmary, Liverpool, UK (16); Royal Manchester Children’s Hospital, Manchester, UK (7); Ludwig-Maximilians University Hospital, Munich, FRG (9); Royal Perth Hospital, Perth Australia (8); King Faisal Hospital, Riyadh, Saudi Arabia (14); University degli Studi di Sapienza, Rome, Italy (39); Medico Marques de Valdecilla, Santander, Spain (34); St Vincent’s Hospital, Sydney, Australia (1); University of Ulm, Ulm, Germany (81).

Total number of patients treated = 951.
Preliminary experience of allogeneic stem cell transplantation for lymphoproliferative disorders using BEAM-CAMPATH conditioning: an effective regimen with low procedure-related toxicity

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Summary. Autologous transplantation has an established role in the treatment of lymphoproliferative disorders, but allogeneic transplantation remains controversial. In an attempt to reduce the high procedure-related mortality reported with allografting in lymphoma, we have used BEAM (BCNU, etoposide, cytarabine and melphalan), a standard conditioning regimen for autologous transplantation. As BEAM may be insufficiently immunosuppressive to permit durable engraftment in the allogeneic setting, patients received additional pretransplant immunosuppression with the anti-CD52 antibody CAMPATH-IG from day -5 to day -1.

Twelve patients (median age 46 years) underwent allogeneic transplantation for lymphoma (n = 11) or chronic lymphocytic leukaemia (n = 1) from HLA-identical (n = 9) or mismatched (n = 3) sibling donors. Cyclosporin A and methotrexate were used as graft-versus-host disease (GVHD) prophylaxis. One patient died of progressive lymphoma at day +12, the remaining 11 patients engrafted rapidly, with eight demonstrating full donor chimerism. One patient had an episode of rejection and received a further stem cell infusion with sustained recovery. Only one patient developed GVHD (grade I). The low incidence of acute GVHD may be in part related to persisting levels of in vivo CAMPATH-IG at the time of transplantation. Of 11 evaluable patients, nine achieved complete remission (CR), and a further patient achieved CR after donor lymphocyte infusion at 5 months.

Our preliminary experience is that this regimen was well tolerated with a low risk of GVHD and appears no more toxic than a BEAM autograft. Further follow-up is required to see whether the low incidence of GVHD impacts upon relapse risk.

Keywords: lymphoma, allogeneic transplantation, BEAM, CAMPATH.

The use of high-dose therapy and autologous haemopoietic stem cell transplantation has a clearly established role in the treatment of Hodgkin's disease and non-Hodgkin's lymphoma (NHL) (Reece et al. 1994; Philip et al. 1995; Freedman et al. 1996). In contrast, the place of allogeneic transplantation in lymphoma is controversial as reported studies both from single centres and from registries have shown a high transplant-related mortality (TRM). Thus, a TRM of 34\% was reported in a series of patients with Hodgkin's disease undergoing allogeneic BMT using conditioning with busulphan/cyclophosphamide or cyclophosphamide/totai body irradiation (TBI) (Jones et al. 1990). In a report from Seattle of 53 patients with Hodgkin's disease undergoing allogeneic BMT, 28 (53\%) suffered non-relapse deaths (Anderson et al. 1993). Reports from the International Bone Marrow Transplantation Registry (IBMTR) and the European Bone Marrow Transplant Group (EBMT) on allogeneic transplantation for Hodgkin's disease have shown a 3-year TRM in excess of 60\%, leading to the conclusion that in most circumstances this procedure could not be recommended (Gajewski et al. 1996; Milpied et al. 1996). A high TRM has also been reported in NHL. In an EBMT series in which the majority of patients received TBI-based conditioning regimes, the TRM for the allograft group was...
25% compared with 11% for the autologous group (Chopra et al., 1992). Recently, a multicentre study of allogeneic BMT in 111 patients with advanced low-grade lymphoma reported a TRM of 40% (Van Besien et al., 1998). A similar high TRM has been seen using the non-TBI-based conditioning (Demirer et al., 1995) CBV regimen (cyclophosphamide, carbamustine and etoposide).

Some studies have suggested that the risk of relapse may be reduced compared with patients undergoing autologous transplantation, possibly as a result of a graft-versus-lymphoma effect (Jones et al., 1990). In NHL, a prospective study of allogeneic compared with autologous transplantation reported a relapse rate of 13% in the autologous arm compared with 51% in the autologous arm (Rakanatharathorn et al., 1994). In low-grade lymphoma, a low rate of recurrence (16%) was reported by Van Besien et al. (1998), although the overall survival was only 49% for a high TRM. A similar low probability of relapse compared with autologous transplantation was reported by Verdonck et al. (1997) in low-grade lymphoma.

The use of potentially less toxic conditioning regimes has been explored by several groups, although the number of reports in the literature remains relatively small. Single agent, melphalan (200 mg/m²) has been shown to permit engraftment in patients with leukemia undergoing allogeneic transplantation from HLA-identical siblings (Singhal et al., 1996). Although the BEAM regimen (BuCVe, etoposide, cytarabine and melphalan) has been widely used as a conditioning protocol for autologous transplantation for lymphoma, there is little experience in its use for allogeneic transplantation. Van Besien et al. (1995) reported on a series of three patients who received BEAM before allogeneic transplantation and demonstrated engraftment in all cases. More recently, a report of a phase II study of BEAM conditioning before autologous transplantation in 30 patients with lymphoid malignancies revealed it was well tolerated, although the incidence of grade II–IV acute graft-versus-host disease (GVHD) was 31% (Frezia et al., 1998). Here, we report our experience of allogeneic transplantation using BEAM conditioning combined with pretransplant CAMPATH-Ig, which was used as additional immunosuppression to facilitate engraftment in recipients of both HLA-matched or one antigen disparate sibling transplants and to provide additional anti-lymphoma activity.

**Patients and Methods**

**Patients.** Between January 1997 and April 1999, 12 patients with NHL (n = 9), Hodgkin’s disease (n = 2) or chronic lymphocytic leukemia (CLL; n = 1) underwent allogeneic transplantation using peripheral blood stem cells (PBSCs: n = 10) or bone marrow (n = 2) from HLA-identical (n = 9) or one antigen mismatched (n = 3) sibling donors. Patients (see Table I) were selected to undergo allogeneic transplantation because of extensive bone marrow involvement (n = 10), peripheral blood pancytopenia (n = 1) or poor prognostic features (n = 1).

**Transplant regimen.** The BEAM treatment was as follows: BCNU (300 mg/m²) on day −6; cytosine arabinoside (200 mg/m²) on days −5 to −2 inclusive; etoposide (200 mg/m²) on days −5 to −2 inclusive; melphalan (140 mg/m²) on day −1. CAMPATH-1G (10 mg) was given intravenously on 5 consecutive days before transplant (days −5 to −1 inclusive). PBSCs mobilised with granulocyte colony-stimulating factor (G-CSF) (filgrastim; 10 µg/kg/d) were used in 10 cases and bone marrow (BM) in two patients, one of whom was one of the recipients of a mismatched graft. GVHD prophylaxis was with cyclosporine (CSA) and methotrexate (MTX) (Byrne et al., 1998) with the exception that methotrexate (10 mg/m²) was given only on days +1, +3 and +6. Donor lymphocyte infusions (DLIs). Donor leukocytes were harvested by leukapheresis on a Fenwal CS3000 cell separator and the CD3+ count was assessed by flow cytometry using a FACScan/Libur (Becton Dickinson) and the CellQuest software. The cells were reinfused fresh or cryopreserved at −80°C in 10% DMSO for subsequent reinfusion.

**Chimerism studies.** Chimerism was studied using four VNTR regions (TNFα, p53[CA], D6S264 and Rb1). Donor/recipient pairs were screened using each microsatellite locus to find at least one informative primer set which was then used to determine the degree of chimerism after transplantation. After amplification, the PCR products were separated on an acrylamide gel using the ABI Prism 377 Automated DNA Sequencer. Gene-Scan Analysis software was used to calculate the peak area of alleles as a quantitative measure of chimerism. The sensitivity of detection using this method is 0.1% (Milin et al., 1999).

**Minimal residual disease (MRD).** MRD monitoring using

<table>
<thead>
<tr>
<th>Table 1: Patient characteristics.</th>
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<tr>
<td><strong>Patient characteristics</strong></td>
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<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Age at transplant</td>
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<tr>
<td><strong>Histology at diagnosis</strong></td>
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<tr>
<td>Follicular lymphoma</td>
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<tr>
<td>Marginal zone lymphoma</td>
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<td>Mantle cell lymphoma</td>
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<tr>
<td>Hodgkin’s disease</td>
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<tr>
<td>Diffuse large-cell lymphoma</td>
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<tr>
<td>Chronic lymphocytic leukemia</td>
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<tr>
<td><strong>Disease at transplant</strong></td>
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<td>Status</td>
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<td>CR</td>
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<tr>
<td>PR</td>
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<td>Resistant/progressive</td>
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<td>Prior CR</td>
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<td>Prior chemotherapy regimes</td>
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<td>Disease duration (months)</td>
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<td><strong>Transplant characteristics</strong></td>
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<tr>
<td>HLA matching</td>
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<tr>
<td>Fully matched</td>
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<tr>
<td>One antigen mismatch</td>
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<td>Donor–recipient sex match</td>
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PCR was undertaken on those patients in whom a molecular marker of disease (either clonal IgH gene rearrangement or presence of the t(14:18) translocation) had been assigned at presentation. PCR for the IgH framework III or t(14:18) major breakpoint region translocation was performed after transplantation and was defined as residual disease by the presence of the same sized products as the diagnostic specimens.

Serum levels of CAMPATH-1G. Serum levels of CAMPATH-1G were measured in 12 patients; in all cases antibody was given from day -5 to day -1. Serum levels were collected before antibody treatment, 1 h after the final dose (peak level), immediately before transplant and 1 h after transplant. The samples were stored frozen at -25°C and heat inactivated at 56°C for 30 min before analysis to destroy any complement activity (tests have shown that this treatment does not damage the antibody). Antibody concentrations were measured by indirect immunofluorescence with a CD52+ T-cell line (HUT-78). The cells were incubated at 4°C for 30 min with test samples or standards serially diluted in PBS containing 0.1% bovine serum albumin (BSA). The cells were washed five times with PBS and then incubated with the detection reagent FITC-labelled anti-mat IgG (Sigma; F-6258). They were washed again and were fixed with 1% formaldehyde before analysis by flow cytometry. The mean fluorescence was measured and antibody concentrations were calculated by interpolation from a standard curve constructed using a reference batch of the therapeutic antibody. The whole experiment was carried out in duplicate and the mean results were used. The overall coefficient of variation was 17%. CAMPATH-1G is a rather low-affinity antibody and the limit of quantification (defined as the concentration that gave fluorescence 50% above background) was 0.13 μg/ml.

RESULTS

Engraftment, GVHD and toxicity

One patient with primary refractory Hodgkin's disease died of disease progression at day +12. The remaining 11 patients who engrafted achieved neutrophils of >0.5 x 10⁹/l at a median of 16 d (range 12-29) and platelets of >20 x 10⁹/l at a median of 21 d (range 14-35). One patient had graft failure at day +28 and required a further PBS infusion from the same donor which resulted in sustained engraftment. The conditioning therapy was well tolerated with minimal mucositis and no significant renal, pulmonary or hepatic toxicity. The median post-transplant in-patient stay was 16 d (range 14-25 d). Only one patient, who was the recipient of a one-antigen mismatched transplant, developed GVHD (grade I). With a median follow-up of 12 months (range 6-32), no patient has developed chronic GVHD. Cytomegalovirus (CMV) reactivation by PCR was seen in two patients, both of whom had mismatched

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Sex/age</th>
<th>Disease at transplant</th>
<th>Stem cell source</th>
<th>Chimerism</th>
<th>Outcome</th>
<th>MRD studies</th>
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<tr>
<td>HD</td>
<td>M/23</td>
<td>PR</td>
<td>PBSC/1-0</td>
<td>100% D</td>
<td>CCR (12 months)</td>
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<td>M/49</td>
<td>Res</td>
<td>PBSC/4-1</td>
<td>100% D</td>
<td>PR; died 6 months</td>
<td>PCR +3 months (14:18)</td>
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<tr>
<td>FL</td>
<td>M/47</td>
<td>PR</td>
<td>PBSC/6-9</td>
<td>60% D 6 months</td>
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<td>90% D 9 months</td>
<td>CCR* (19 months)</td>
<td>PCR +3,6,9,12,17,19 months (14:18)</td>
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<td>95% D 12 months</td>
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<tr>
<td>MCL</td>
<td>M/54</td>
<td>PR</td>
<td>BM/0-7</td>
<td>100% R</td>
<td>CCR (18 months)</td>
<td>No marker</td>
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<tr>
<td>MZL</td>
<td>F/49</td>
<td>PR</td>
<td>PBSC/2-4</td>
<td>100% D</td>
<td>CR; died 6 months</td>
<td>PCR +3 months (igG)</td>
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<tr>
<td>FL</td>
<td>M/48</td>
<td>Res</td>
<td>PBSC/1-4</td>
<td>100% D</td>
<td>CCR (15 months)</td>
<td>PCR +3 and 10 months (14:18)</td>
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<tr>
<td>HD</td>
<td>M/36</td>
<td>Prog</td>
<td>PBSC/5-0</td>
<td>N/E</td>
<td>Died day 12</td>
<td>N/E</td>
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<tr>
<td>FL</td>
<td>M/40</td>
<td>PR</td>
<td>PBSC/4-4</td>
<td>100% D</td>
<td>CCR (11 months)</td>
<td>PCR +3 and 9 months; PCR +6 months (14:18)</td>
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<tr>
<td>FL</td>
<td>M/44</td>
<td>PR</td>
<td>PBSC/5-1</td>
<td>75% D 1 month</td>
<td>CCR (9 months)</td>
<td>PCR +3 months; PCR +6 months (14:18)</td>
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<td>&lt;20% D 4 months</td>
<td>&lt;20% D 7 months</td>
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<tr>
<td>MCL</td>
<td>M/49</td>
<td>PR</td>
<td>PBSC/1-6</td>
<td>100% D</td>
<td>CCR (8 months)</td>
<td>PCR +3 and 6 months (igH)</td>
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<tr>
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<td>F/41</td>
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<td>PBSC/6-1</td>
<td>100% D</td>
<td>CCR (7 months)</td>
<td>PCR +3 months (igH)</td>
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<tr>
<td>DLCL</td>
<td>M/37</td>
<td>Res</td>
<td>PBSC/3-4</td>
<td>100% D</td>
<td>CCR (5 months)</td>
<td>No marker</td>
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* Patient received DLI at 15 months for persistent bcl-2 rearrangement.
† Patient had localized inguinal node relapse at 10 months that was treated with excision, radiotherapy and DLI.
‡ Patient received DLI for residual marrow infiltration.

PCR, partial remission; CR, complete remission; CCR, continuing complete remission; Res, resistant disease; Prog, progressive disease; D. donor DNA; R, recipient DNA; N/E, not evaluable; MRD, minimal residual disease. See Table I for diagnosis definitions.
transplants but neither developed CMV disease. One recipient of a mismatched transplant died from para-influenza pneumonia at 6 months after the transplant while in remission.

**Disease response**

At the time of transplant, eight patients were in partial remission and four had resistant or progressive disease (Table II). At 3 months after transplantation, all patients were assessed by computerized tomography (CT) scan and bone marrow analysis. Of 11 evaluable patients, nine had achieved a complete remission at this stage. Two patients with follicular lymphoma achieved a partial remission (PR) at 3 months, with evidence of persisting bone marrow infiltration on trephine biopsy. Of these, one had an overt relapse at 6 months with rapid progression and death despite intervention with DLIs. The other patient received DLI (5 × 10⁷ CD3+ cells/kg) at 5 months, and when assessed at 6 months by CT and bone marrow was in clinical and molecular remission according to PCR for t(14;18). One patient with follicular lymphoma developed a localized nodal relapse that was treated with radiotherapy and DLI (2 × 10⁷ CD3+ cells/kg) and remains in remission with a short follow-up. Another patient with follicular lymphoma who was in clinical remission but persistently positive by PCR for t(14;18) in bone marrow aspirates received DLI (5 × 10⁶ CD3+ cells/kg) at 15 months and remains PCR positive 4 months later. Currently, 9 out of 12 patients are alive, of whom eight remain event free with a median follow-up of 12 months. The overall survival and disease-free survival of the patients is shown in Figs 1 and 2.

**Chimerism**

Of 11 evaluable patients, eight had full donor chimerism when assessed at 3 months after transplantation and two patients had evidence of mixed chimerism (see Table II). The remaining one patient, who received BM rather than PBSCs, has 100% recipient DNA at 6 months but remains in complete remission at 18 months after the transplant.

![Cumulative Survival](image)

**Fig 1.** Event-free survival.

![Overall Survival](image)

**Fig 2.** Overall survival.

**Minimal residual disease**

Of eight patients with evidence of a clonal population of B cells in the marrow immediately before transplant, the result of either an IgH or a bcl-2 rearrangement, five achieved a molecular remission (Table II). One patient with follicular lymphoma who achieved a PR with a persistent bcl-2 rearrangement entered clinical and molecular remission after receiving DLI therapy, whereas another patient who achieved CR remains PCR positive at 19 months despite DLI therapy at 15 months.

**Serum levels of CAMPATH-1G antibody**

Antibody levels were measured to establish whether there was residual CAMPATH-1G at the time of transplant that might contribute to depletion of donor T cells. The peak concentration on day −1, 1 h after the last infusion of antibody, was 1.8 ± 1.1 μg/ml (mean ± SD). The stem cell infusion started 17 ± 3 h later and, at that point, the serum antibody concentration was 0.9 ± 0.6 μg/ml in 10/12 patients but <0.13 μg/ml in two patients. At 1 h after the stem cell infusion (24 ± 4 h after the peak antibody level), the serum antibody concentration was 0.7 ± 0.6 μg/ml in 6/11 patients and <0.13 μg/ml in 5/11 patients (one sample was not collected). The antibody half-life under these conditions was ∼13 h. All antibody levels were well below the concentration required for saturation binding to cells *in vitro* (∼25 μg/ml), but the mean level observed at the time of bone marrow infusion would still be sufficient to mediate a substantial degree of cell killing by antibody-dependent cell-mediated cytotoxicity (ADCC) (Hale et al., 1987).

**DISCUSSION**

This paper describes our preliminary experience of the BEAM regimen for allogeneic transplantation in lymphoproliferative disorders. The BEAM regimen has been extensively used before autologous transplantation and it is well tolerated with low incidence of side-effects and a TRM of <10%
(Chopra et al., 1993). We reasoned that BEAM would be a suitable conditioning regimen for allogeneic transplantation. However, because of concerns that BEAM might not be sufficiently immunosuppressive to permit engraftment in the allogeneic setting, particularly when using mismatched family members, we added pretransplant CAMPATH-1G. There are other theoretical advantages relating to the use of CAMPATH before transplant in the lymphoproliferative disorders as the CD52 antigen recognized by the CAMPATH antibodies is heavily expressed on lymphoid neoplasms. Thus, CD52 is expressed on 100% of low-grade B-cell NHL, the majority of high-grade B-cell NHL and a significant proportion of T-cell lymphomas (Salsbury et al., 1994). Indeed, CAMPATH-1H, the humanized version of CAMPATH-1G, is currently being evaluated as therapy in CLL (Osterborg et al., 1997). B-prolymphocytic leukemia (Bowen et al., 1997) and low-grade NHL (Landin et al., 1998).

This protocol aimed to use PBSC where possible to obtain a high stem cell dose to facilitate engraftment when using a regimen that might not be as immunosuppressive as TBI-based conditioning regimens and that is not truly myeloablative, as exemplified by reports of haemopoietic recovery after BEAM without stem cell support (LaPorte et al., 1991). In this study, we used PBSC in 10 patients, two donors preferring to give bone marrow. All evaluable patients achieved sustained haemopoietic engraftment, although one patient required a second infusion of PBSCs at day +28. Also, one recipient of a mismatched transplant had autologous reconstitution, and it may be relevant that this patient received BM rather than PBSC. The BEAM–CAMPATH regimen was well tolerated with minimal non-haemopoietic toxicity. This low toxicity contrasts with the results obtained using CBV as conditioning for allogeneic BMT in lymphoma (Demirer et al., 1995) in which there was a high TRM due primarily to pulmonary toxicity, which was seen particularly in those patients who received doses of BCNU of 600 mg/m². The dose of BCNU conventionally used in BEAM is only 300 mg/m², and a low incidence of pulmonary toxicity has been seen after autologous transplantation using this dosage (Chopra et al., 1993).

No cases of acute GVHD higher than grade 1 were seen. A low incidence of GVHD using CAMPATH-1G as part of the conditioning protocol was reported, particularly if the antibody was continued after transplantation (Hamblin et al., 1996). In our study, CAMPATH-1G was given up to day 1 before transplant (i.e. day –11) and, to assess the presence of residual antibody at the time of transplant, serum levels were analysed 1 h after the final dose (peak), immediately before transplant and 1 h after transplant. The levels of antibody measured immediately before transplant were found to be sufficient to mediate a substantial degree of ADCC and, therefore, killing of infused T cells in the majority of patients (Hale et al., 1987). Furthermore, the serum levels observed after transplant were still sufficient to mediate ADCC in approximately half of the patients. However, the levels were less than half of those seen directly after antibody infusion, and the T cell depletion would likely be less effective than if the CAMPATH-1G was continued after the transplant. Nonetheless, it is probable that there was purging of donor T cells by residual CAMPATH-1G using this protocol, and this may have been sufficient to abrogate the development of significant GVHD. It is also possible that the use of BEAM may be associated with a lower GVHD risk because of reduced tissue damage and hence of cytokine release compared with TBI-based regimens. We used GVHD prophylaxis with CSA and MTX, although it has been our policy to discontinue CSA at 3–4 months and it is possible that further reductions in immunosuppression can be made in order to maximize any graft-versus-lymphoma effect.

Eight of the 12 patients transplanted in our study had low-grade lymphoma, and in a recent analysis of 64 patients undergoing allogeneic transplantation for lymphoma this subgroup was found to have the most favourable outcome with the lowest rate of disease recurrence (Van Besien et al., 1996). That these results could be improved by a reduction in TRM has led to the development of minitransplant regimens that have been predominantly fludarabine based (Khouri et al., 1998). Although perhaps not a true minitransplant regimen, BEAM–CAMPATH has a significantly lower toxicity profile than that seen with TBI-based regimens.

DLIs were administered to four patients, all of whom had follicular lymphoma. One patient received a DLI for residual disease after transplant and entered clinical and molecular remission. A second patient who achieved CR but remained persistently PCR positive for (14;18) for more than a year has not entered a molecular remission 6 months after DLI therapy. One patient who relapsed at 6 months progressed rapidly and died despite DLI therapy. A fourth patient who had a localized relapse at 10 months was treated with radiotherapy and DLIs and remains in remission, although the follow-up is short. Thus, DLIs have proven effective in at least one patient and our current approach is to consider DLIs for patients not in CR at 3 months or who are in clinical CR but remain PCR positive at 12 months.

In conclusion, our preliminary experience suggests that the toxicity of BEAM–CAMPATH is similar to that seen after autologous transplantation. An additional advantage of this regimen is the fact that fertility, at least in some women, is maintained (Chopra et al., 1993). There has been a striking low incidence of acute and chronic GVHD and longer follow-up will be required to determine whether this is maintained and whether this has an impact upon relapse risk. Because of the low toxicity that we have observed, we are proposing to extend its use in patients aged up to 65 years.

REFERENCES


Allogeneic Transplantation in Lymphoma with BEAM-CAMPATH 759


T cell depletion by exposure to Campath-1G in vitro prevents graft-versus-host disease

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Summary:

Campath-1G is an immunosuppressive monoclonal antibody directed against human lymphocytes. Its effectiveness in preventing graft-versus-host disease (GVHD) by simple opsonisation of bone marrow T-cells has been studied in 36 consecutive allografts: in 17 for leukaemia, one for essential thrombocytosis and four for myeloma, this was the sole means of GVHD prophylaxis. A further eight patients with aplastic anaemia received 3 months post-transplantation cyclosporin A (CsA) for this purpose whereas in the ninth and tenth the preparative regimen has been modified with this immunosuppressive agent now discontinued. Nucleated cells were harvested and after quantitative recovery of the mononuclear population on the Cobe 2997 separator they were exposed to 20 mg Campath-1G for 30 min at room temperature and then infused. Following standard conditioning, which included total lymphoid irradiation, the median days to reach 0.5 and 1.0 x 10⁹/l neutrophils were respectively 18 (range 9–34) and 28 (range 10–59); to 25 and 100 x 10⁹/l platelets the corresponding times were 17 days (range 5–32 days) and 27 days (range 13–127 days). In all, the day 14 trephine biopsy showed engraftment. At median follow-up of 20 months (range 5–44 months) only one patient has developed possible grade I cutaneous GVHD that responded promptly to corticosteroids: no chronic GVHD or CMV pneumonitis has been encountered. Of those with haematological malignancy transplanted in remission only two with acute leukaemia have relapsed. In aplastic anaemia graft loss initially occurred but this has been overcome by adding Campath-1G in vivo and omitting CsA. These results, although preliminary, show that in vitro opsonisation by an antibody can virtually abolish GVHD.

Allogeneic BMT is potentially curative for patients with aplastic anaemia,1 immunodeficiency diseases2 and certain congenital enzymphathies.3 It has also been employed as a means of intensification therapy for a variety of malignant tumours arising from the haematopoetic system.4–9 The majority of these procedures are complicated by acute or chronic GVHD which have a high morbidity and substantial mortality.10,11 Attempts to reduce both its incidence and severity have included post-transplant immunosuppression with MTX, cyclosporin A (CsA), combinations of the two or even more complex regimens.12,13 None of these approaches has been uniformly successful. Furthermore, it has been suggested that the occurrence of GVHD can be justified on the basis that there is an associated reduction in leukaemic relapse.14 However, the devastation wrought by the more advanced grades of GVHD appears to offset these benefits so that survival rates are not significantly different.15 Unfortunately in such analyses inappropriately little weight is given to the discomfort inflicted on the recipients.16

In contrast to the more conventional immunosuppressive regimens came a different approach based on experimental work in a variety of animal models where it was shown that removal of mature T lymphocytes from the graft could reduce GVHD. Clinical studies, using a variety of mechanical methods or mAbs, met with variable success.16 Historically the use of an opsonising antibody with the intent that the coated cells be removed in the host was mooted more than a decade ago although this manoeuvre was of limited benefit with the antibody used.17 The development of a rat monoclonal immunoglobulin that fixes human, as opposed to rabbit, complement and could react with all lymphocytes, while sparing colony-forming cells, suggested that it would be a useful reagent in BMT.18 Experience has confirmed a significant reduction in acute and chronic GVHD.19 However, an associated problem was the increased incidence of graft failure resulting in a number of deaths. Such rejection was encountered more frequently with mismatched donors but rarely in autologous transplants employing the same purging technique20 with the conclusion that alloreactive recipient T lymphocytes can be implicated in its pathogenesis and so raising the question whether extra immunosuppression might not override this complication.21

More recently, the rat IgG2b mAb Campath-1G became available and this has the important characteristic of fixing human complement as well as binding to Fc receptors, thereby providing the capacity to clear
coated lymphocytes in vivo. This feature is potentially attractive in that it leads to depletion of those residual helper or CD4 lymphocytes which are refractory to conditioning by total body irradiation (TBI) and combination chemotherapy. In some studies Campath-1G has been given to the recipient but, alternatively, marrow cells can be exposed to its action in vitro. We have now established that this treatment can lead to the virtual prevention of acute and chronic GVHD in patients having haematological malignancy or aplastic anaemia with the additional important modification that no post-transplant immunosuppression was given in the former group, initially only 3 months therapy with CsA in the latter and this practice has now also been discontinued.

Patients and methods

Patients

Thirty-two individuals received 36 allogeneic transplants from HLA-identical and MLR non-reactive siblings in a programme approved by the Ethics and Research Committee of the University of Cape Town Groote Schuur Hospital (Table I).

Table I Details of the 32 patients undergoing 36 T-depleted allogeneic bone marrow transplants

<table>
<thead>
<tr>
<th>Patient</th>
<th>Donor</th>
<th>Status</th>
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<tbody>
<tr>
<td><strong>AML</strong></td>
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<tr>
<td>1.</td>
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<tr>
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<tr>
<td>3.</td>
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<tr>
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<td>30 F</td>
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<tr>
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<td><strong>Essential thrombocythaemia</strong></td>
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<td><strong>Severe aplastic anaemia</strong></td>
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<tr>
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<tr>
<td>36.</td>
<td>38</td>
<td>21 F</td>
</tr>
</tbody>
</table>

a. = second transplant; AIDS = acquired immunodeficiency syndrome; APL = acute progranulocytic leukaemia; b. = third transplant; CCR = continuous complete remission; CNS = central nervous system; DC = disease controlled; FSP = first stable phase; NPTI = no post-transplant immunosuppression; PCP = Pneumocystis carinii pneumonia; PP = plateau phase; PTI = post-transplant immunosuppression with CsA; S = heavily sensitised; VOD = veno-occlusive disease
Prevention of GVHD with Campath-1G

Acute leukaemias
Patients with AML underwent one induction and two consolidation courses with a combination of Ara C, daunorubicin and etoposide. In ALL, the regimen comprised Ara C, etoposide and MTX with follicic acid rescue. Once in CR they received total body irradiation (TBI) in fractions of 2 Gy twice a day on days -10 to -8, CY (60 mg/kg with uromexan (mesna) cover on days -6 and -5 and 1.5 Gy total lymphoid irradiation (TLI) twice a day on days -3 and -2.

Chronic granulocytic leukaemia and essential thrombocythaemia
Disease control was rapidly achieved with hydroxyurea after which the same conditioning regimen was employed.

Myeloma
Plateau phase of the disease was induced with pulses of CY, vincristine, adriamycin (doxorubicin hydrochloride) and steroid after which a priming dose of 400 mg/m² of CY was given on day -12. 2 Gy of TBI twice a day on days -10 to -8 followed by 140 mg/m² of melphalan on day -6 and then 1.5 Gy of TLI twice a day on days -3 and -2.

Post-transplant immunosuppression was not employed in these three groups with haematological malignancy.

Aplastic anaemia
On admission, CY 50 mg/kg was given, under uromexan (mesna) cover, on days -12 to -9 followed by 1.5 Gy of TLI twice a day on days -7 to -2.

Three patients lost their established grafts and were reconditioned with the same CY regimen but TLI was replaced with an iv regimen comprising 30 mg/kg of antilymphocyte globulin (Swiss Serum Institute, Bern, Switzerland) on days -4 to -2 combined with 10 mg of Campath-1G on days -5 to +4 in two patients, and inadvertently omitted in the third. This individual again rejected and was successfully re-transplanted after preparation with TBI.

CSA, as a single agent titrated to a whole blood assay level of 200 ± 50 ng/ml, was used for 3 months in the first eight patients but, based on the stable haematopoiesis achieved in the regrafts with additional in vivo moAb, it has been discontinued.

Supportive management
All individuals were in laminar downflow units and prior to conditioning commenced on selective decontamination of the gastrointestinal tract with 200 mg twice daily of oral ofloxacin. They also received 200 mg of oral acyclovir for five times in 24 h and cotrimoxazole two tablets twice a day on Mondays and Thursdays. Antibiotics were given only for confirmed fever: where this was of undetermined origin ceftazidime 1 g every 6 h was combined with gentamicin 3 mg/kg with therapeutic peak and trough levels maintained using antibiotic assays.

Bone marrow collection technique
For every kilogram of recipient lean body mass, 15 ml of BM was aspirated from the sternum and iliac crests into 1 ml of Iscove's modified Dulbecco tissue culture medium containing 10 units of preservative-free heparin for each millilitre of graft collected. A mononuclear suspension was achieved by gently passing this material through a series of stainless steel screens; cell loss was <1% and retention of viability 100%, as defined by Trypan blue exclusion.

Technical details of graft manipulation
Recovery and concentration of stem cells
The marrow-rich blood and the tissue culture medium was circulated through a Cobe 2997 blood cell separator as previously described with recovery of 100 ± 10% of haematopoietic progenitors using the CFU-GM assay.

The absence of mononuclear cells in the discard cells was confirmed and these reinfused into the donor to reconstitute red cell mass. There were no complications and all patients were discharged from hospital within 24 hours of admission.

BM infusion technique
The median total number of nucleated cells harvested was 4.1 × 10⁹/kg (range 2.3–8.0). After separation the middle volume of autologous plasma was 128 ml (range 105–180) which contained a median of 1.3 × 10⁹/kg mononuclear cells (MNC range 0.32–2.8) corresponding to 3.04 × 10⁷ T lymphocytes/kg (range 1.5–5.8); 20 mg of Campath-1G was added to this suspension, giving a ratio of 2.5 µg/10⁶ MNC cells. After gentle agitation at room temperature (± 2°C) for 30 min the entire contents of the bag were given iv to the recipient under cover of premedication with 100 mg of iv hydrocortisone and 25 mg of phenegran. Full non-invasive monitoring was used during this period. Fever was the only symptom occurring with approximately 40% of infusions but no changes in oxygen saturation or cardiovascular status were documented.

Statistics
Kaplan–Meier analysis was used to determine the probability of overall and disease-free survival for the entire group and, separately, for those with haematological malignancy or aplastic anaemia.
Results

Engraftment interval

In all procedures, including the four re-transplants, haematopoiesis was evident on day 14 by trephine biopsy. The median time to 0.5 and 1.0 x 10^9/l granulocytes were, respectively, 18 (range 9-34) and 28 (range 10-59) days and the corresponding intervals to reach 25 and 100 x 10^9/l platelets were 17 (5-32) and 27 (13-127) days.

The numbers are too small for subgroup analysis but between those with malignancy and aplastic anaemia no differences are currently discernible.

GVHD

A single patient with myeloma developed a rash which was consistent with grade I GVHD. It responded promptly to corticosteroids. No chronic GVHD has occurred.

Malignant disease

Eight of the nine patients with AML transplanted in CR remain continuously disease-free: the ninth patient, in second CR with the acute progranulocytic variant, relapsed at 1 year and after re-induction with retinoic acid, is again in CR; the tenth patient, inadvertently grafted before CR was achieved, died. Of the three with high risk lymphoblastic leukaemia, one is in CR and two are dead: one in CR of AIDS and the other, with multiple cytogenetic abnormalities, from recurrence.

All four patients with stable phase CGL and one with essential thrombocythaemia are without haematological or cytogenetic evidence of disease to date. Of the four with myeloma, the one referred to above as having GVHD, with an excellent graft but scaly skin, died from fulminating *Pneumocystis carinii* pneumonia, since he believed that the minimal rash was due to cotrimoxazole which he discontinued: the other three remain in CR.

Severe aplastic anaemia

One of the first eight in this group had been multiply transfused over 6 years prior to referral, remained pancytopenic and died of a stroke. The next seven had established haematopoiesis by day 14 but three suffered graft failure within 3 months and were successfully re-transplanted from the same donor but on a revised regimen that combines Campath-1G ‘in-the-bag’ with additional *in vivo* administration but CsA is no longer used. In these two individuals engraftment has been particularly swift and complete.

Outcome

There was no direct transplant-related mortality or development of CMV pneumonitis. With median follow-up at 20 months (range 5-44 months) the actuarial probability of survival for the entire group is 81% and for the same population, censored for disease-unrelated deaths, reflecting freedom from relapse, is 91%. For those 22 with haematological malignancies the corresponding figures are 81% and 91% and for the ten with aplastic anaemia are 80% and 90% (Figure 1).

Discussion

Even in matched siblings acute and chronic GVHD remain the most significant barrier preventing BMT from realising its full therapeutic potential. Attempts to abrogate morbidity and mortality with a number of post-transplantation immunosuppressive interventions have been only modestly successful. MTX, with or without CsA, is of benefit but does not eliminate GVHD. A wide range of moAb, used either *in vivo* or *in vitro*, has been described but no regimen has gained general acceptance.

It is therefore of interest that our preliminary data with exposure of mononuclear cells to Campath-1G *in vitro* appear to have the unique characteristic of almost completely preventing this complication. A single patient had a mild flaky skin rash whereas none has this far developed any evidence of chronic GVHD. Of note has been the absence of CMV pneumonitis, which may be an additional benefit derived from abrogating GVHD. If these results can be confirmed it would make matched sibling transplants more widely acceptable. Furthermore, it will provide an encouraging base

![Figure 1](image-url)  
**Figure 1** Kaplan-Meier analysis. Probability of survival for all 32 patients (A) and the same data censored for disease-unrelated deaths reflecting freedom from relapse (B). The corresponding curves for the groups with malignancy (n = 22) or aplasia (n = 10) are not shown separately since they are the same and both superimpose on those for the total population (n = 32). Ticks identify individuals at risk.
from which to explore varying degrees of mismatch with the recipient, whether this be from alternative family members or unrelated donor programmes.

With regard to AML in CR a single relapse occurred in one patient with APL; a third remission was readily achieved with vincristine and he is alive and well. In patients with high-risk ALL one with multiple cytogenetic abnormalities developed CNS disease and died. A longer period of observation and larger numbers are essential to define the incidence of disease recurrence but so far the antibody treatment has not had any significant adverse effect. Five patients with chronic myeloproliferative diseases also remain in remission. Here, while the benefits of avoiding GVHD are obvious, no useful statement can be made about the possible future re-emergence of the neoplastic clone. Cytogenetic studies have not, thus far, demonstrated the return of the Philadelphia chromosome in the four patients with CGL. It is possible that the intensified conditioning associated with the use of total nodal irradiation to overcome the increased risk of graft rejection may, concurrently, offset any loss of an anti-leukaemic effect from GVHD and some evidence supports this concept.33

Among the four allografts for myeloma, one patient was thought to have minimal cutaneous acute GVHD. However, it could equally well have been caused by the cotrimoxazole and unfortunately when the patient chose to discontinue this agent he developed Pneumocystis carinii pneumonia resulting in his death. The other three patients have not shown recurrence of their tumour.

The situation with severe aplastic anaemia is encouraging in that no GVHD has been encountered. It is nevertheless disappointing that, despite the initial use of post-transplant immunosuppression with CsA, four biospy-proven grafts were lost, so that repeat procedures were needed. This contrasts with the low rejection rate reported in similar cases, whether T cell depleted or not44 and irrespective of previous transfusions.34,35 The benefit attributed to the use of TLI. The reason for this discrepancy is not clear but it may reflect the small numbers in each of the series.

Additionally, while regrafting was uncomplicated and hospitalisation relatively brief this is a cost-ineffective sequence of events. In contrast, the modification used in the last two patients that combines Campath-1G 'in-the-bag' with its parenteral administration but omitting CsA, resulted in the rapid reconstitution of peripheral values in the most cellular grafts seen on the day 14 biopsies and is therefore our standard approach to allogeneic transplantation in severe aplasia. It is noteworthy that the level of 25 × 10^9/L platelets was reached on day 16 which is even earlier than the enhanced marrow recovery achieved when part of the graft is pre-incubated with IL-3 and GM-CSF.36

One caveat attending the use of irradiation in young individuals is the potential for the subsequent development of malignant disease.37 This issue remains controversial with the advantages of TNI being appreciated both with transplantation35,34,45 and in those who lack a donor.41 at least in one series, no neoplasia has occurred to date.35 Furthermore, there is the observation that aplasia, managed with regimens containing no radiation, is also associated with similar problems of clonal haematopoietic abnormalities including paroxysmal nocturnal haemoglobinuria and leukaemia.37,42

Overall, we believe that these results are most encouraging. If confirmed, they have at least three potentially important implications. Firstly, they offer a significant advantage in that acute and chronic GVHD in sibling grafts can be virtually prevented by exposure to Campath-1G 'in-the-bag'. If this benefit can be transferred to incompatible family members or matched unrelated donors both options might become more widely used and expenditure in operating BM donor registries more realistic. Secondly, in allografting for malignant disease no further intervention is necessary but in aplasia additional in vivo antibody favours rapid stable engraftment. In both of these circumstances short-term morbidity and mortality as well as hospital stay and financial costs, are reduced in parallel with eliminating the inconvenience and dangers of post-transplantation immunosuppression. Thirdly, programmes free of GVHD and graft failure may allow new drug or immunological regimens to be evaluated for anti-leukaemic activity.

It is concluded that the mAb Campath-1G, added to mononuclear cells in vitro and without further CsA or MTX, is a major advance in allogeneic BMT. The rapidity of engraftment, the failure to develop acute or chronic GVHD and the low frequency of leukaemic relapse to date are encouraging: we still need to study larger numbers of patients and to extend the period of follow-up.

Acknowledgements

We thank our nursing and medical colleagues for excellent help with patient care, Dr Carol S. Johnson and her staff for radiotherapy, Vincent Parker for help with statistical analysis, Wayne Jacobs for the graphs, Christine Dolling and Jessica Gerenseth for bibliographic assistance and Mrs Di Jacobs for help with the preparation and typing of the manuscript. This work was supported by the University of Cape Town Leukaemia Centre and Staff Research (Becker, Cancer and Foote) Fund, the Gwendoline Moore Trust, the National Cancer Association, the Medical Research Council, the Michael Chanani, M.A. Richardson and Kaliski Bequests, the Kay Kendall Trust, Leukaemia Research Fund and Medical Research Council, UK.

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Adhesion molecules play an important role in the engagement and mobilization of stem cells during peripheral blood stem cell transplantation (PBSC). Colony stimulating factors are widely used for mobilization of stem cells. During mobilization stem cells may induce some changes in the adhesion molecule expression of the stem cell and stroma which will end in the release of soluble adhesion molecules. To demonstrate this we measured the levels of various adhesion molecules in the sera of healthy donors who were primed with G-CSF (Neupogen, Roche) for allologeneic PBSC transplantation. Fifteen healthy donors (7 male and 8 female) were given 10μg/kg/day s.c. granulocyte colony-stimulating factor for 5 days. Peripheral blood stem cell collection was performed on the fifth day two hours after the last dose of G-CSF. Serum ICAM-1, E-Selectin, L-Selectin and sCD44 levels were detected by using a commercial ELISA Kit (Bender Med, Austria) before, on the third day and 24 hours after the last dose of G-CSF. The results indicate a steady rise in the levels of sCD44, sE-Selectin, sL-Selectin, and sICAM-1 after G-CSF administration, reaching a maximum after the last dose of G-CSF (p<0.05 for all instances). There is no change in the levels of sICAM (p>0.05). Further analysis will be presented to see if there is any correlation with CD44 levels and leukocyte and platelet engraftment times. This results may provide a clue to the role of adhesion molecules in stem cell mobilization but the correlation between the soluble adhesion levels and rising count of neutrophils due to G-CSF administration yet remains to be determined.

A RANDOMIZED DOUBLE BLIND TRIAL OF T-CELL DEPLETION WITH CD8/30 CELLLECTOR IN ADDITION TO CYCLOSPORIN, METHOTREXATE AND STEROIDS FOR ACUTE GRAFT VERUS HOST DISEASE PREVENTION IN HLA MISMATCHED BONE MARROW TRANSPLANTATION. J. Galantowicz, D. Wall, D. Adams, R. Geller, A. Yver, K. Lloyd, S. Souza, R. Chandola, and the ASBMR Study Group. UT MI, Anderson Cancer Center, Houston, TX; St. Louis University, St. Louis, MO; Washington University, St. Louis, MO; Emory University, Atlanta, GA; RPR Gencell, Santa Clara CA, USA.

Acute graft versus host disease (GVHD) is a major cause of mortality in mismatched (MM) bone marrow transplantation (BMT). T-cell depletion (TCD) reduces GVHD but increases infection and rejection. 61 patients treated with MM BMT using total body irradiation, cyclophosphamide, thiopeta, antithymocyte globulin with cyclosporin, methotrexate and steroids for AGVHD prophylaxis were randomized for partial TCD. Stratification is reviewed below.

**TRANSPANT TYPE**

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<th>TCD CONTROL</th>
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<tr>
<td>Unrelated Donor</td>
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<tr>
<td>Mismatched Unrelated Donor</td>
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</tbody>
</table>

**DISEASE STAGE**

| Good Risk Disease | 17 |
| Poor Risk Disease | 14 |

**AGE (mean)**

(51, 51)

Good risk disease was CML in first chronic phase or acute leukemia in 1 st to 2 nd complete remission. Results are reviewed below.

**TCD CONTROL**

| Good (0.05) | 0.05 |
|------------|
| 0.7 (0.05) | 0.7 |
| 0.02 (0.05) | 0.02 |

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**EX VIVO T-CELL DEPLETION WITH CAMPATH 1G COMPLETELY ABROGATES GRAFT-VERSUS-HOST DISEASE (GVHD) IN PERIPHERAL BLOOD STEM CELL (PBSC) ALLOGRAFTS. J. Jacoby, L. Wood, G. Haig, H. Wiertman. Haematology Department, Wythenshawe Hospital, South Africa; and Sir William Dunn School of Pathology, Oxford, England.

Transplantation of unmanipulated CD34+ bone marrow from HLA identical sibling donors provides rapid and stable hematopoietic recovery, but also does not preclude the use of GvHD prophylaxis. To further reduce incidence of acute GVHD, immunoselected CD34+ blood cell grafts were additionally T-cell depleted with Campath-1H (C-1H) in seven patients (3 CML, 2 AML, 2 NHL, median age 36 years, range 25-47, 3 male, 4 female). The conditioning regimen included TBI (120Gy) and Cy (120mg/kg). C-1H was given i.v. prior to conditioning for prophylaxis of graft rejection (100mg, d-11 to -7). At transplantation the CD34+ cells were thawed and C-1H was added (10μg/100 μl). Median cell numbers were 4.7 x 10^9/μg CD34+ and 6.9 x 10^9/μg CD3+ cells >95% of the T-cells were labelled with C-1H. The patients received G-CSF (5μg/kg, s.c.) post-transplant. No further GvHD prophylaxis was given. Nine patients engrafted. No graft failure or rejection were observed so far (5 follow-up). Median recovery time of neutrophils to reach 500 and 1,000/μl was 13 and 14 days, respectively. Median time to reach 25,000/μl platelets was 16 days (50,000/μl, 24). None of the patients developed GVHD. While neutrophils engrafted more rapidly compared to control patients receiving CD34+ blood cell grafts without additional treatment, no patient receiving and had no GVHD. Forty patients received cryopreserved donor T cells from the CD34 negative fraction on days 0 and 100. 0.1 x 10^9 CD3+ cells/kg, because of a switch to better PRA-screening patients developed a mild GVHD and entered molecular remission. The transplantation of closely T cell allogeneic CD34+ blood cells prevented effectively aGVHD, preserving the rapid hematopoietic reconstitution seen with PBSC grafts. Since no immunosuppressive treatment is used post-transplant, this approach provides appropriate conditions for induction of GVHD with donor lymphocyte transfusions.
In vivo CAMPATH-1H prevents graft-versus-host disease following nonmyeloablative stem cell transplantation


A novel nonmyeloablative conditioning regimen was investigated in 44 patients with hematologic malignancies. The median patient age was 41 years. Many of the patients had high-risk features, including 19 women with previous failed transplant. Recipient conditioning consisted of CAMPATH-1H, 20 mg/day on days −8 to −4; fludarabine, 30 mg/m² on days −7 to −3; and melphalan, 140 mg/m² on day −2. Thirty-six recipients received unmanipulated granulocyte colony-stimulating factor–mobilized peripheral blood stem cells from HLA-identical siblings, and 8 received unmanipulated marrow from matched unrelated donors. GVHD prophylaxis was with cyclosporine A alone for 38 patients and cyclosporine A plus methotrexate for 6 sibling recipients. Forty-two of the 43 evaluable patients had sustained engraftment. Results of chimeraism analysis using microsatellite polymerase chain reaction indicate that 18 of 31 patients studied were full-donor chimeras while the other patients were mixed chimeras in one or more lineages. At a median follow-up of 9 months (range 3 to 29 months), 33 patients remain alive in complete remission or with no evidence of disease progression. Seven patients relapsed or progressed post-transplantation, and 4 of them subsequently died. Four patients died of regimen-related complications.

Introduction

High-dose chemoradiotherapy followed by allogeneic stem cell transplantation (SCT) has been extensively used to treat patients with hematologic malignancies. This procedure is often limited to patients in good medical condition because of the increased risk of regimen-related toxicity and graft-versus-host disease (GVHD) that occurs with increasing age and poor performance status. The curative potential of transplantation is not solely due to the conditioning regimen but also to the well-documented graft-versus-leukemia (GVL) effect. The most convincing evidence for this GVL effect is that donor leukocyte infusions (DLIs) can reinduce remissions in patients who have relapsed following allogeneic SCT. Patients with chronic myeloid leukemia are most likely to respond, but responses have also been documented in patients with acute leukemia. Chronic lymphocytic leukemia, myeloma, and lymphoma. In an effort to reduce the transplant-related mortality (TRM) associated with allogeneic SCT, low-intensity fludarabine-based regimens have been developed. These have been designed to be immunosuppressive rather than myeloablative to facilitate donor engraftment and thereby limit systemic toxicity. There appears to be a spectrum of hematopoietic toxicity associated with these nonmyeloablative regimens—from minimally cytopenic regimens that use low-dose total body irradiation alone to regimens that combine fludarabine with melphalan or busulfan. While these studies have demonstrated impressive allogeneic engraftment with minimal nonhematologic toxicity, there is still a significant morbidity and mortality from acute and chronic GVHD.

We have therefore developed a novel nonmyeloablative regimen for allogeneic SCT. Our regimen was designed to suppress the recipient immune system enough to allow allogeneic engraftment without excessive regimen toxicity or GVHD. The use of fludarabine as an immunosuppressant as part of the conditioning regimen was similar to previously published studies of nonmyeloablative SCT. The combination of fludarabine with 180 mg/m² of melphalan was originally described by the M. D. Anderson group. Our regimen used a different dose of melphalan, 140 mg/m²; however, the addition of an in vivo CAMPATH-1H to the conditioning regimen was new and appears to have been crucial in limiting graft-versus-host reactions.
Patients and methods

Eligibility criteria

Patients with hematologic malignancies were enrolled at 6 hospitals in England. The study design was approved by the ethics committees at each participating site. All patients gave written informed consent to participate. Patients with lymphoma, acute leukemia, myelodysplasia, multiple myeloma, chronic lymphocytic leukemia, and chronic myeloid leukemia between ages 18 and 60 years were eligible to participate. Patients required an HLA-identical sibling or unrelated donor as determined by serologic typing for HLA-A/B and molecular typing for HLA DR/DQ. Data were analyzed as of November 30, 1999.

Patient characteristics

Detailed characteristics are shown in Table 1. Forty-four patients were enrolled in the study from June 1997 to September 1999. Twenty-eight were male, and 16 were females. Age range at the time of transplantation was 18 to 56 years (median, 41 years). Fourteen patients had non-Hodgkin lymphoma (NHL). 10 Hodgkin disease, 6 acute myeloid leukemia (AML), 7 multiple myeloma, 3 hypoplastic myelodysplastic syndrome, 1 acute lymphoblastic leukemia, 1 chronic lymphocytic leukemia, 1 chronic myeloid leukemia, and 1 plasma cell leukemia. This was a cohort of patients with high-risk features, including 19 patients with a previous failed autologous (18 patients) or allogeneic (1 patient) transplant, relapse failure (2 patients), poor left ventricular function (2 patients), or refractory disorder (8 patients). The median time interval from first to second transplant was 24 months (range, 6-79 months).

Table 1. Patient characteristics, source of progenitor cells, and GVHD prophylaxis (n = 44)

<table>
<thead>
<tr>
<th>Patient no.</th>
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MCL indicates mantle cell lymphoma; HD, Hodgkin disease; AML, acute myeloid leukemia; LG-NHL, low-grade non-Hodgkin lymphoma; T, transformed low-grade to high-grade lymphoma; MDS, myelodysplastic syndrome; HG-NHL, high-grade non-Hodgkin lymphoma; MM, multiple myeloma; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; CMN, chronic myeloid leukemia; CR, complete remission; PR, partial remission; CP, chronic phase; PBSC, peripheral blood stem cells; MUD, matched unrelated donor; CsA, cyclosporine A; MTX, methotrexate; TBI, total body irradiation.
Monoclonal antibody

CAMPATH-1H is a humanized immunoglobulin (Ig) Gl monoclonal antibody against the CD52 antigen. It was prepared from the culture supernatant of Chinese hamster ovary cell transfectants cultured in a hollow fiber fermentor. It was purified by affinity chromatography on protein A-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, England) and size exclusion chromatography on Superdex 200 (Amersham Pharmacia Biotech) and formulated in phosphate-buffered saline. The half-life of CAMPATH-1H in humans is dependent on the amount of target CD52 antigen in the patient. Based on work in progress, there is persistence on CAMPATH-1H in vivo past day 0 sufficient to cause T-cell lysis by antibody-dependent cell-mediated cytotoxicity.

Conditioning regimen

Treatment consisted of the humanized monoclonal antibody CAMPATH-1H, 20 μg/kg intravenously infusion over 8 hours on days -8 to -4; fludarabine, 30 mg/m² intravenous infusion over 30 minutes on days 7 to 3; and melphalan, 140 mg/m² intravenous infusion over 30 minutes on day -2. Thirty-six recipients received unmanipulated peripheral blood stem cells (PBSCs) from their siblings, and 8 received unmanipulated marrow from matched unrelated donors.

Stem cell and bone marrow collection

Sibling donors received granulocyte colony-stimulating factor (G-CSF) at 10 μg/kg subcutaneously once daily on days -4 to 0. Leukaphereses were performed on days 0 and +1 using conventional techniques for PBSC collection. Unrelated donors had bone marrow collected on day 0 under general anesthesia using conventional techniques. In 2 sibling donors, we failed to collect more than 2 × 10⁷/kg CD34+ cells from G-CSF-mobilized peripheral blood, and therefore bone marrow was also harvested. The total number of CD34+ cells collected from peripheral blood and the number of mononuclear cells collected from the bone marrow are shown in Table 1. Unmanipulated mobilized peripheral blood or bone marrow was infused through central venous catheters on days 0 and +1 and on day 0, respectively.

Supportive care

Patients were managed in reverse isolation in conventional or laminar airflow rooms. All patients received prophylaxis with cotrimoxazole or pentamidine against Pneumocystis carinii infection. Aseptolir and fluconazole or itraconazole prophylaxis were routinely used. Blood products were irradiated to 25 Gy. Red cell and platelet transfusions were given to maintain hemoglobin levels above 9 g/dL and platelet count above 10 to 15 × 10⁹/L. The cytomegalovirus (CMV)-seronegative patients received only CMV-negative blood products; seropositive patients received CMV-unscreened blood products. Fibrile neutropenic patients received broad-spectrum intravenous antibiotics according to each hospital's policy for the management of neutropenic sepsis. G-CSF at 5 μg/kg per day was administered subcutaneously at the discretion of the transplant physician to speed hematologic recovery in 38 patients until the patient's absolute neutrophil count was at least 1000/μL for 3 consecutive days (Table 2).

GVHD prophylaxis and grading

GVHD prophylaxis consisted of cyclosporine A (CsA), 3 mg/kg starting on day -1, and methotrexate (MTX) at a dose of 10 mg/m² on days +1, +3, and +6 for 6 sibling recipients, and it consisted of CsA alone for the other 38 patients. Intravenous CsA was switched to an oral dose as soon as the patients would tolerate medications by mouth and was continued for a median of 4 months (range, 1 to 8 months). Patients who survived 100 days or longer were evaluable for chronic GVHD. Acute and chronic GVHD were graded according to the consensus criteria.³⁶

Follow-up

Patients had regular follow-up at 3 monthly intervals post-transplantation to assess disease response and remission status. These evaluations varied depending on the underlying diagnosis but included bone marrow aspirates or biopsies, cytogenetics, computed tomography scans, paraprotein levels, and skeletal survey.

Chimerism analysis

DNA was prepared from posttransplantation recipient blood and donor blood. Following transplantation, either buffy coat or granulocyte T-cell and B-cell preparations were obtained from peripheral blood as previously described. We used 4 different primers sets each spanning highly polymorphic short tandem repeat units on different human chromosomes. With primers VWA1 (Perkin-Elmer) and TH01 (Perkin-Elmer), polymorphic chain reaction (PCR) volumes were 50 μL containing Genematch PCR buffer II (Perkin-Elmer), 1.5-mmol/L MgCl₂, 0.2 mmol/L of each dNTP, and 0.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Foster City, CA), and 5 μL of DNA. Cycling conditions were 95°C for 45 seconds, 45°C for 30 seconds, and 72°C for 1 minute for 30 cycles. With primers AC17-GCC- CTAATCGTATACCT, backwards AGTGAACGGAAGTCCACCT and HUMSTRX1 (forward CTCCTTTGAGCTCCCTTCTAAAGG, backwards CTCCTAGCAGCCAGGGAAGTCA), PCR volumes were 50 μL containing 10 mmol/L Tris⋅HCl, 11-mmol/L NaH₂PO₄, 6.7 mmol/L, 2.5 mmol/L MgCl₂, 4.5 μmol/L of each dNTP, 1 μmol/L of each primer, 0.5 U of AmpliTaq polymerase (Perkin-Elmer), and 5 μL of DNA. Cycling conditions were 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds for 30 cycles. The forward primer of each pair was labeled with either JOE or FAM fluorescent dyes. One microliter of PCR product was denatured in 12 μL of formamide and electrophoresed through a Performance Optimized Polymer 4 (Perkin-Elmer) on an ABI 373 automated sequencer (Perkin-Elmer) in the presence of Rox 500 size standard (Perkin-Elmer). Genescan software 2.1 (Perkin-Elmer) was used to analyze the data. Primers that gave rise to recipient/donor-specific peaks were identified and used for post-transplantation determination of chimerism status in various cell populations.

Study endpoints

The primary study endpoint was the successful durable hematopoietic engraftment and TRM. There were secondary endpoints, including regimen-related toxicity, incidence and severity of GVHD, and progression-free survival.

Statistical methods

Actuarial curves were estimated according to the Kaplan-Meier method. Surviving patients were censored on the last day of follow-up. The significance of differences between the curves was estimated by the log-rank test. Cox multivariate regression analysis was performed to calculate the independent effects of various risk factors influencing nonrelapse mortality, overall survival, and disease-free survival. The proportional hazards assumption was tested using a time-dependent covariant approach.

Results

Toxicities

All patients were assessable for toxicity. The conditioning regimen was generally well tolerated in patients who only received CsA as GVHD prophylaxis. The use of MTX in addition to CsA was associated with severe mucositis and delayed engraftment (Table 2). The original intention was to give MTX to all patients, but its use was abandoned because of toxicity. There were no cases of veno-occlusive disease. Four patients died of regimen-related toxicity. One patient died on day 121 of gram-negative septicemia while still aplastic. The second patient died on day +24 of idiopathic pneumonitis after engrafting on day +14. The third
Table 2. Transplant outcome of 44 patients receiving a nonmyeloablative regimen

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<th>Platelets &gt; 20×10⁹/L days</th>
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NE indicates nonevaluable; CR, complete remission.
*Patient received MTX as GVHD prophylaxis.
†Patient did not receive post-transplantation G-CSF.

Patient died of MRSA pneumonia on day +153. The fourth patient with myeloma, whose neutrophin clearance prior to transplantation was 23 mL/min, died of renal failure on day +148.

Engraftment

One patient was not evaluable for engraftment because of early death on day +21. Of the 43 patients eligible for assessment of engraftment, 42 had sustained engraftment as defined by neutrophil counts above 0.5×10⁹/L and an untransfused platelet count of above 20×10⁹/L for at least 3 consecutive days. Details of the neutrophil and platelet reconstitution are shown in Table 2. The median time to recover an absolute neutrophil count of 0.5×10⁹/L was 13 days (range, 8-23 days) and of 1.0×10⁹/L was 17 days (range, 8-47 days). The median time to achieve platelets above 20×10⁹/L was 13 days (range, 3-96 days) and above 50×10⁹/L was 17 days (range, 8-118 days). Patient No. 7 developed graft rejection. After initial engraftment on day +11, the patient became cytopenic on day +20 and reconstituted recipient hemopoiesis on day +31 without autologous stem cell support.

Chimerism

Thirty-one patients had chimerism studies performed using microsatellite PCR or fluorescent in situ hybridization for X and Y chromosomes on peripheral blood. Detailed results are shown in
Table 3. Patient No. 7, who rejected his graft, had only recipient myeloid and lymphoid cells present. Of the other 30 patients, 18 had only donor cells present. Of the 12 patients with mixed chimerism, 5 had detailed lineage-specific studies performed using microsatellite PCR. Three of these patients were full-donor chimeras in the myeloid and B-cell lineages but were mixed T-cell chimeras. Two patients were mixed chimeras in all lineages tested (Figure 1). Six of the 8 unrelated recipients had chimerism studies performed, and all 6 were found to have only donor cells present following transplantation (Table 3). Two patients (Nos. 1 and 11) who were mixed chimeras post-transplantation became full-donor chimeras following DLI therapy.

**Graft-versus-host disease**

No grade III-IV acute GVHD was observed post-transplantation. Three patients developed grade I skin, 1 patient grade II gastrointestinal, and 1 patient grade II skin and gastrointestinal (Table 2) acute GVHD. Only 1 patient has developed chronic GVHD, limited to skin and liver involvement. Two patients developed GVHD following DLI to treat relapse of disease. One of these patients had steroid-resistant grade IV acute GVHD, and the other had limited chronic GVHD.

**Disease response and relapses**

Current disease status is shown in detail in Table 2. The conditioning regimen induced remissions in 2 of 2 patients with refractory AML and 2 of 2 evaluable patients with myelodysplasia. Of the 6 patients with Hodgkin disease in partial remission or with refractory disease at transplantation, 2 achieved a complete remission (CR) following transplantation, and 4 are progression-free. Seven patients with NHL in partial remission or with refractory disease prior to transplantation were evaluable for disease response (Table

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Discussion

TRM remains a major obstacle to successful allogeneic SCT. The introduction of nonmyeloablative purine analogue conditioning regimens has facilitated allogeneic engraftment while limiting regimen-related mortality.11,12 Despite this, GVHD remains a significant cause of mortality and morbidity following nonmyeloablative conditioning. Previously published results reporting other nonmyeloablative conditioning regimens have shown a 38% to 60% incidence of grade II-IV acute GVHD.8,11,13 This was the primary cause of death in some patients.

In our study, the incidence of GVHD was exceptionally low. No patients had grade III-IV acute GVHD, and only 2 patients (5%) developed grade II acute GVHD. The incidence of chronic GVHD was also low, with only 1 patient developing limited skin GVHD. While the incidence of chronic GVHD cannot yet be fully assessed in some of the patients because of relatively short follow-up, given the fact that only 1 patient has experienced this complication and that all but 3 of the patients are off all immunosuppression, we anticipate a very low rate of chronic GVHD. Because the use of post-transplantation CsA was similar to other nonmyeloablative regimens, the differences in the incidence and severity of GVHD may in part reflect the in vivo use of the immunosuppressive monoclonal antibody CAMPATH-1H as part of the conditioning regimen.18 This was administered to the patients on days −8 to −4 prior to transplantation. Because CAMPATH-1H has a prolonged half-life in humans, there was significant circulating antibody when the unmanipulated donor PBSCs or bone marrow was infused into the recipient, resulting in a degree of in vivo T-cell depletion. This combination of in vivo CAMPATH-1H together with CsA appears to have been very effective in preventing GVHD in both sibling and unrelated donor allograft recipients. A number of these patients have had their CsA discontinued between 1 and 3 months post-transplantation without the development of GVHD.

While our nonmyeloablative conditioning regimen facilitated engraftment in all but one of the evaluable patients, many of the patients were mixed chimeras. Some patients were mixed chimeras in all lineages tested, while others were only mixed chimeras in the T-cell lineage. It has been demonstrated that patients who are mixed chimeras may experience less GVHD than full-donor chimeras.19,20 On the other hand, mixed chimerism may diminish the potential benefit of the GVL effect seen in the allograft setting.21,22 While mixed chimeras can be converted to full-donor chimeras following DLI,2 this was not attempted as part of this pilot study. The primary end points of this study were to explore the incidence of durable engraftment and acute and chronic GVHD. DLI were only given for overt rejection of disease and were not given prophylactically or pre-emptively because we wished to assess the impact of the conditioning regimen on disease control and relapse. In the present study, we were generally not able to show the benefit of DLI in the setting of post-transplantation
relapse. DLs were either ineffective or led to toxicity from GVHD in all but one of the patients treated. This is not surprising, because the response rate in relapsed AML is low and there are few data to show that DLs are effective in inducing remissions in patients with aggressive lymphomas.23-25 Only a single patient with multiple myeloma has achieved CR following DL therapy.

The use of this conditioning regimen has been very effective in a group of patients that had many high-risk features: patients who had prior high-dose therapy, patients with renal or cardiac impairment, or patients with high-risk diagnoses for allogeneic SCT such as Hodgkin disease or multiple myeloma. Indeed, allogeneic transplantation using myeloablative conditioning following failed autologous transplantation has been associated with a treatment mortality ranging between 50% and 80%.23,26 Undoubtedly, such a high mortality rate may offset a potential for cure, and therefore conventional transplants have generally been avoided in such patients. In our study, 19 patients received a second transplant, and only 3 patients (17%) died of transplantation-related complications, demonstrating that this nonmyeloablative approach could be attempted if a second transplant has to be considered.

While a conditioning regimen containing fludarabine and melphalan appears to have been active in tumor control, particularly in patients with Hodgkin disease and NHL, the follow-up period is still very limited and all patients remain at risk of relapse.

In such a high-risk group of patients, any conditioning regimen is likely to be associated with a significant relapse risk, and therefore the survival curves shown in Figure 2 should be interpreted with caution. This is particularly so in some hematologic malignancies, such as acute leukemia, where the GVL effect of DLI for the treatment of relapse is of limited efficacy.23 However, the antitumor responses seen with the conditioning regimen might allow the use of DLI to be delayed until 6 to 12 months post-transplantation, when this intervention might be associated with less GVHD.21,24

In summary, our results show that our nonmyeloablative regimen facilitates allogeneic engraftment, with a low incidence of GVHD and TRM. The long-term antitumor activity of this regimen remains unknown, however, if used in combination with the prophylactic or pre-emptive use of DLI, prolonged remissions might be obtained in some types of hematologic malignancies.

Acknowledgments

We thank the staff members of the Therapeutic Antibody Centre, University of Oxford, for their contributions to the production of CAMPATH-1H antibody.

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Meeting Report
CAMPATH-1 antibodies in stem-cell transplantation

G Hale1, S Cobbold1, N Novitzky2, D Bunjes3, R Willemze4, HG Prentice5, D Milligan6, S Mackinnon7 and H Waldmann1 on behalf of CAMPATH Users

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6 Department of Haematology, Heartlands Hospital, Birmingham, UK
7 Department of Haematology, University College Hospital, London, UK

Background
CAMPATH-1 (CD52) Abs have been used in stem-cell transplants for the prevention of GvHD and graft rejection. These complications can effectively be prevented by depletion of T lymphocytes from both donor and recipient. However, donor lymphocytes might contribute to an anti-leukemia effect and lymphocyte depletion may exacerbate problems with immune reconstitution. There is a fine balance between the risks of GvHD and host-versus-graft reactions, relapse and infection.

Methods
Clinical outcomes for 4264 patients were reported to a central registry and analyzed by univariate and multivariate methods to determine the superior protocols. Various protocols of lymphocyte depletion were tested, using either CAMPATH-1M (lgM) plus complement or CAMPATH-1G (lgG2a) to treat the donor BM ex vivo and CAMPATH-1G in vivo to treat the recipients. The humanized antibody CAMPATH-1H has recently replaced CAMPATH-1G. A meeting of the clinical collaborators was convened to discuss the results and to review the experiences of individual centers.

Results
Interest focused on the use of mobilized PBSC for transplantation and on the use of reduced-intensity conditioning regimens ('mini' or 'non-myeloablative' transplants). These approaches are likely to become increasingly important in the future and will allow transplant procedures to be used for relatively older patients. The use of CAMPATH-1G or CAMPATH-1H was associated with a low incidence of GvHD or rejection, though there were some differences that might be related to the longer half-life of the humanized antibody. An unexpected and apparently paradoxical effect of post-transplant CYA was observed — it appeared to reduce the risk of dying from infection after 6 months. Although part of the benefit could be explained by a reduction in GvHD, the effect was still evident when patients with GvHD or graft rejection were excluded from analysis.

Discussion
CAMPATH-1H appears to have a useful role in the prevention of graft rejection and GvHD, particularly in patients who are at high risk of these complications. It can equally well be used by admixture with the infused stem cells, or by administration to the patient prior to the transplant. Future studies will seek to understand the mechanism of the CYA effect and to improve the quality of immune reconstitution.

Keywords
CAMPATH, CD52, PBSC, bone marrow, cyclosporin, T-cell depletion, registry.

Correspondence to: Geoff Hale, Therapeutic Antibody Centre, Old Road, Headington, Oxford OX3 7JT, UK.

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Introduction

International clinical investigators met in Oxford UK in September 2000, to review the results of clinical studies using CAMPATH-1 Abs for immunosuppression in stem-cell transplantation. They had previously submitted updated results to the CAMPATH Users’ registry and this report summarizes the statistical analysis of those data and discussion that took place at the meeting. This is a registry-type analysis and, as such, has the advantages and drawbacks inherent in that type of study [1]. By pooling data from many centers it is possible to answer questions that it would be hard to address even by very large prospective randomized trials. However, this approach requires accurate and complete data collection and there is a risk that variables will be confounding, leading to erroneous conclusions about causal relationships. The CAMPATH users have made strenuous efforts to provide complete and up-to-date data for analysis, and we have developed algorithms for multifactorial analysis of this type of dataset.

The Campath family

The group has worked with three different CAMPATH-1 Abs [2]. They all recognize the same epitope on the CD52 Ag, a small lipid-anchored glycoprotein that is found on almost all lymphocytes. The original CAMPATH-1M (rat IgM) is the most efficient for complement activation and has been used exclusively in vitro for treatment of donor stem cells with exogenous complement. CAMPATH-1G (rat IgG2b) does activate complement, but also binds human Fc receptors and lysed cells very effectively in vivo. It has been used both in vivo, to treat the recipient before and/or after the transplant, and also in vitro, to treat the stem cells. When lymphocytes are coated with CAMPATH-1G, there is a small and variable degree of lysis, probably depending on the amount of residual plasma in the cell suspension, but most of the cells are presumed to be lysed when the mixture is infused into the patient. A key feature of this treatment is that excess CAMPATH-1G is also likely to deplete residual recipient lymphocytes. CAMPATH-1H is a humanized IgG1, created from CAMPATH-1G by genetic engineering [3].

Antibody protocols

The main protocols for using CAMPATH-1 Abs are shown in Figure 1. In protocols 01–04 the donor BM is depleted of T cells in vitro, with or without CAMPATH-1G in vivo to control rejection. In protocols 06–07, CAMPATH-1G has been used in vivo alone, before and/or after the transplant. Since 1997, CAMPATH-1H has been used to replace CAMPATH-1G in protocols 03, 04, 06 and 07, and one object of the analysis was to see whether the clinical results were comparable.

Conclusions from previous meetings

The following topics were not discussed in detail since the current analysis generally confirms earlier published results [4,5].

- T-cell depletion of donor BM with CAMPATH-1M or CAMPATH-1G results in:
  - Reduction in risk of GvHD;
  - Increase in risk of graft rejection;
  - Increase in risk of relapse (a large effect in CML, small in acute leukemia).

The risk of graft rejection can be reduced by additional immunosuppression, including:

- Additional conditioning with TLI;
Additional conditioning with CAMPATH-1G.
- Post-transplant CYA.

Attempts to prevent or treat relapse by:
- Adding back small numbers of donor T cells increases GvHD with a marginal effect on the risk of relapse.
- Pre-emptive donor lymphocyte infusions can be very effective in treating CML following molecular or cytogenetic relapse, but there is a risk of severe GvHD.

Combination of CAMPATH-1 Abs in vivo and in vitro can sometimes delay engraftment.

Patients and methods
Patient recruitment: exclusion and inclusion for analysis
The registry collects basic enrolment and outcome data for all patients who receive treatment with CAMPATH-1 Abs as part of conditioning or GvHD prophylaxis for stem-cell transplants. From the correlation between antibody distribution records and registry returns, we estimate that the database includes at least 98% of all patients treated during 1982–99. Between April and August 2000, updated data were received from all of the 38 currently active transplant centers, representing 88% of the patients in the registry.

A total of 4764 allogeneic transplants have been reported. Of these, 202 were excluded from analysis, mostly because of short follow-up (< 100 days), or because CAMPATH-1 was used in conjunction with other methods for T-cell depletion, or they were from centers contributing only one or two patients. Sixty-one centers contributed to the final dataset of 4062 transplants, of which 3922 were first allogeneic transplants and 140 were second or subsequent (Table 1).

No attempt was made to classify the degree of HLA matching for unrelated donor transplants, since precise information was not available for all the patients, especially those transplanted before the era of molecular genotyping. Notwithstanding the likely effects of HLA matching on the outcomes, all of the unrelated donor transplants have been considered together. All types of graft failure, early, late or partial, whether or not successfully treated, were included together and percentages were calculated as a fraction of patients at risk (i.e. survived long enough to be evaluable for engraftment). To calculate the risk of GvHD, patients who suffered graft failure without GvHD were considered not evaluable and, again, the percentages were calculated as a fraction of those 'at risk'. Only hematologic relapse was analyzed; for patients with CML there was no account of molecular or cytogenetic relapse since the diagnostic criteria have changed over the time scale of these studies.

Univariate statistical analysis
Treatment groups were compared using the χ² test for categorical variables and the Wilcoxon two-sample test for continuous variables. Life-table analyses were carried out by the standard method using the LOGRANK computer software specially developed for this purpose (Steve Cobbold, unpublished work). This program can display the estimated standard error of the survival curves at each event point [6], which allows a simple visual estimation of significance. It is empirically determined that if there is 'daylight' between the error zones of two curves, then the difference is usually statistically significant (at least

<table>
<thead>
<tr>
<th></th>
<th>Matched sibling</th>
<th>Unrelated donor</th>
<th>Related donor</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total reported</td>
<td>2101</td>
<td>1679</td>
<td>484</td>
<td>4264</td>
</tr>
<tr>
<td>Excluded from analysis</td>
<td>73</td>
<td>83</td>
<td>46</td>
<td>202</td>
</tr>
<tr>
<td>First marrow transplant</td>
<td>1563</td>
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<td>336</td>
<td>3439</td>
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<td>First PBSC transplant</td>
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<tr>
<td>Second or combined transplant</td>
<td>61</td>
<td>38</td>
<td>41</td>
<td>140</td>
</tr>
<tr>
<td>Analyzed</td>
<td>2028</td>
<td>1596</td>
<td>438</td>
<td>4062</td>
</tr>
</tbody>
</table>
Multivariate statistical analysis
The dataset of 3922 first-transplant patients was analysed by Cox multivariable regression model, using the custom software package COXSURV (Steve Cobbold, unpublished work) to automatically carry out the necessary iterations. First, the data were coded appropriately, either binary (male/female, in vivo antibody/no in vivo antibody etc.) or in numeric groups (e.g. age, log cell dose etc.). Data were checked for confounded variables and, as expected, several were found (e.g. total irradiation dose + number of fractions). The worst of these were eliminated from subsequent analysis. The factors that remained are shown in Table 2. The first univariate iteration was carried on each of the covariates. The factor with both the highest relative risk (i.e. making the biggest contribution to the outcome) and a significant probability value was then fixed and all other variables recalculated. This was repeated until there were no more significant variables. The program can plot 'corrected' survival curves. These were modeled for any particular variable, or pairs of variables, allowing for the contribution of all other significant variables. In a standard life-table, group survival is calculated thus

\[ P_n = \frac{P_{n-1} (AtRisk_n - \text{events}_n)}{(AtRisk_n - \text{Censored events}_n)} \]

where:
\[ AtRisk_n = N - \sum_1^n \text{events} \]
\[ N = \text{total number of patients in the group} \]

In the multivariable model, accumulated risk exposure replaces accumulated patients numbers and is calculated as follows:

\[ P_n = P_{n-1} \frac{(AtRisk_n - \text{Risk}_n)}{(AtRisk_n - \text{Censored Risk}_n)} \]

where:
\[ \text{Risk}_n = \text{the product of individual risk factors, i.e. the sum of the Log_e(Risks)} \]
\[ \text{i.e.:} \]
\[ \text{Risk}_n = E + \beta_1(a_n - mn[a]) + \beta_2(b_n - mn[b]) + \ldots \]
\[ E = 1 \text{ if an event occurs. } E = 0 \text{ if there is no event} \]
\[ a, b, \ldots \text{ are significant variables included in the model} \]
\[ mn[a], mn[b], \ldots \text{ are the means of } a, b, \ldots \]

Table 2. Parameters used in multivariable analysis after exclusion of confounded variables

<table>
<thead>
<tr>
<th>Factor</th>
<th>Coding for Cox model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Match</td>
<td>1 = matched sib, 2 = matched family, 3 = unrelated, 4 = mismatch family</td>
</tr>
<tr>
<td>Age</td>
<td>Actual age/10</td>
</tr>
<tr>
<td>Patient gender</td>
<td>0 = female, 1 = male</td>
</tr>
<tr>
<td>Disease status</td>
<td>0 = non-malignant, 1, 2, 3 = complete remission or chronic phase, 4 = partial remission, 5 = refractory or CML-accelerated, 6 = relapse or CML-blast crisis</td>
</tr>
<tr>
<td>Donor gender</td>
<td>0 = female, 1 = male</td>
</tr>
<tr>
<td>Source of stem cells</td>
<td>0 = BM, 1 = peripheral blood</td>
</tr>
<tr>
<td>Cell dose</td>
<td>Log_{10}(mononuclear cell dose)</td>
</tr>
<tr>
<td>Type of conditioning</td>
<td>0 = including cyclophosphamide and TBI, 1 = any other</td>
</tr>
<tr>
<td>TBI dose</td>
<td>Dose in Gy over 10Gy</td>
</tr>
<tr>
<td>CAMPATH-1M</td>
<td>0 = none, 1 = CAMPATH-1M in vitro</td>
</tr>
<tr>
<td>CAMPATH-1G</td>
<td>0 = none, 1 = CAMPATH-1G in vivo and/or in vitro</td>
</tr>
<tr>
<td>CAMPATH-1H</td>
<td>0 = none, 1 = CAMPATH-1H in vivo and/or in vitro</td>
</tr>
<tr>
<td>Ab pre-transplant</td>
<td>0 = none, 1 = any CAMPATH pre-transplant</td>
</tr>
<tr>
<td>CYA</td>
<td>0 = none, 1 = cyclosporin post-transplant</td>
</tr>
<tr>
<td>MTX</td>
<td>0 = none, 1 = methotrexate post-transplant</td>
</tr>
<tr>
<td>T-cell add-back</td>
<td>0 = none, 1 = T cells added back to T-depleted transplant</td>
</tr>
</tbody>
</table>
βₐ is the covariate coefficient from the Cox model associated with variable a
β for the variable(s) being investigated are set to zero.

\[ \text{AtRisk}_n = \sum_{i=1}^{N} \text{Risk}_i - \sum_{i=1}^{n} \text{Risk}_i, \]

i.e. accumulated risk exposure replaces accumulated patient numbers.

To handle missing data points, unknown variables are set to the mean and the risk from patients where the variable of interest is not known is distributed equally between the two groups. Risk factors can be estimated for each variable as:

\[ \text{Riskfactor}_i = e^{(\beta_i \times \text{mean})}. \]

**Results — univariate analysis**

**Overview**

Results for each protocol were divided into two groups: with and without post-transplant CYA (± MTX). As became apparent, there was often a substantial difference between them. Outcomes were classified according to protocol, disease, source of stem cells (BM or blood) and type of donor (HLA-identical sibling or unrelated). The numbers of mismatched family donor transplants were too small for detailed analysis by protocol.

**HLA-matched siblings — BMT**

Total data for all diseases are presented in Table 3. There is some bias because different protocols may have been used for different diseases. Therefore, separate analyses were carried out for non-malignant diseases (Table 4), patients with acute leukemia in first CR (Table 5), and CML in first chronic phase (Table 6). Relatively few patients had received CAMPATH-1H, so these were pooled with the corresponding CAMPATH-1G data in most cases. However, comparison of CAMPATH-1H and CAMPATH-1G was possible for Protocol 07 (CAMPATH-1G/1H pre-transplant), where it appeared that the results were essentially similar whichever was used (Table 4).

The protocols that gave the best control of both GvHD and rejection, and the lowest transplant mortality, were Protocol 02 (CAMPATH-1G plus CAMPATH-1M) and Protocol 03 (CAMPATH-1G ‘in the bag’). Post-transplant CYA gave a reduction in transplant mortality that often seemed to be greater than its effect on GvHD or rejection. This effect was predominantly due to a difference in deaths from infection that occurred between 6 months and 2 years, and was still evident even when patients who suffered any GvHD or graft rejection were excluded from analysis (Figure 2).

Results from three centers who used Protocol 02 to treat patients with AML in first remission were published [7]. Comparison with case-matched controls treated with conventional CYA/MTX GvHD prophylaxis, as reported to the International Bone Marrow Transplant Registry (IBMTR) showed that the CAMPATH-treated group did significantly better. It is not possible to continue with this approach due to lack of commercial interest in production of the rat Abs.

The use of CAMPATH-1G or CAMPATH-1H pre-transplant (Protocol 07) with post-transplant CYA, gave a low incidence of acute and chronic GvHD, comparable with the other protocols. Antibody levels have been measured in a small number of patients transplanted from unrelated donors, and these results suggest that there is sufficient antibody in the recipient at the time of marrow infusion to deplete the donor T cells. CAMPATH-1H may have a longer half-life than CAMPATH-1G in this context, since it was still detectable for several days post-transplant [8].

**HLA-matched siblings — PBSC transplants**

Most patients have followed Protocol 03 (CAMPATH-1G/1H in the bag) — generally with full conditioning including TBI. A significant number of patients were treated using Protocol 07 (CAMPATH-1G/1H pre-transplant) — most of these were ‘mini’ transplants where fludarabine/melphalan were used as conditioning agents.
### Table 3. HLA-matched sibling BMT: all diseases (malignant and non-malignant)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patient</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Remit %</th>
<th>Survival %</th>
<th>Leuk-free survival %</th>
<th>Transplant mortality %</th>
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<tbody>
<tr>
<td></td>
<td>in vivo</td>
<td>in vitro</td>
<td></td>
<td>500 neut day Yes No</td>
<td>0/1 2 3/4</td>
<td>O M S</td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>-</td>
<td>1M</td>
<td>+ CYA</td>
<td>255</td>
<td>20</td>
<td>85 15</td>
<td>87 7 6</td>
<td>78 21 2</td>
<td>19</td>
<td>63 ± 4</td>
<td>56 ± 3</td>
<td>46 ± 3</td>
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<td>-</td>
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<td>- CYA</td>
<td>306</td>
<td>19</td>
<td>80 20</td>
<td>81 12 7</td>
<td>84 15 2</td>
<td>26</td>
<td>64 ± 4</td>
<td>38 ± 3</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>+ CYA</td>
<td>33</td>
<td>22</td>
<td>97 3</td>
<td>89 7 4</td>
<td>92 8 0</td>
<td>6</td>
<td>80 ± 8</td>
<td>71 ± 8</td>
<td>66 ± 9</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>- CYA</td>
<td>195</td>
<td>22</td>
<td>91 9</td>
<td>89 10 1</td>
<td>79 21 0</td>
<td>9</td>
<td>68 ± 4</td>
<td>57 ± 4</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>-</td>
<td>1G</td>
<td>+ CYA</td>
<td>32</td>
<td>18</td>
<td>81 19</td>
<td>100 0 0</td>
<td>96 4 0</td>
<td>19</td>
<td>77 ± 10</td>
<td>69 ± 10</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>-</td>
<td>1G</td>
<td>- CYA</td>
<td>128</td>
<td>22</td>
<td>93 7</td>
<td>93 4 3</td>
<td>88 6 6</td>
<td>13</td>
<td>72 ± 5</td>
<td>65 ± 4</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>1G</td>
<td>1G</td>
<td>+ CYA</td>
<td>29</td>
<td>15</td>
<td>89 11</td>
<td>83 17 0</td>
<td>95 0 5</td>
<td>14</td>
<td>66 ± 11</td>
<td>51 ± 10</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>1G</td>
<td>1G</td>
<td>- CYA</td>
<td>95</td>
<td>27</td>
<td>80 20</td>
<td>95 4 1</td>
<td>99 1 0</td>
<td>21</td>
<td>64 ± 6</td>
<td>57 ± 5</td>
<td>47 ± 5</td>
</tr>
<tr>
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<td>17</td>
<td>88 12</td>
<td>89 8 3</td>
<td>94 6 0</td>
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<td>83 ± 6</td>
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<tr>
<td>1G/H</td>
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<td>- CYA</td>
<td>34</td>
<td>22</td>
<td>94 6</td>
<td>67 17 17</td>
<td>52 17 30</td>
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<tr>
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<td>+ CYA</td>
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<td>87 13</td>
<td>76 12 11</td>
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<td>25</td>
<td>93 ± 3</td>
<td>80 ± 5</td>
<td>78 ± 5</td>
</tr>
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</table>

1 A high proportion of the patients in this category (96) were transplanted for non-malignant conditions (see Table 4).
<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patient Marrow (in vivo)</th>
<th>Total cases</th>
<th>500 neut day</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad (%)</th>
<th>Actuarial at 2 years (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<td>No</td>
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<td>6</td>
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<td>1G +CYA</td>
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<tr>
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<td>1G +CYA</td>
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<td>87</td>
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<td>87</td>
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<td>89</td>
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Table 5. HLA-matched sibling BMT: acute leukemia in first remission (ALL-CR1 and AML-CR1)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases</th>
<th>500 day neut</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
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<td></td>
<td>Remit</td>
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<td>20</td>
<td>20</td>
<td>95</td>
<td>5</td>
<td>83</td>
<td>6</td>
<td>11</td>
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</table>
Table 6. HLA-matched sibling BMT: chronic myeloid leukemia in first chronic phase (CML-CP1)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patient</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases</th>
<th>500 neut</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>(in vivo)</td>
<td>(in vitro)</td>
<td></td>
<td>n</td>
<td>day</td>
<td>Yes</td>
<td>No</td>
<td>0/1</td>
<td>2</td>
<td>3/4</td>
</tr>
<tr>
<td>-</td>
<td>1M</td>
<td>+CYA</td>
<td>88</td>
<td>21</td>
<td>86</td>
<td>14</td>
<td>86</td>
<td>8</td>
<td>6</td>
<td>71</td>
</tr>
<tr>
<td>-</td>
<td>1M</td>
<td>-CYA</td>
<td>67</td>
<td>21</td>
<td>82</td>
<td>18</td>
<td>77</td>
<td>17</td>
<td>6</td>
<td>86</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>-CYA</td>
<td>44</td>
<td>20</td>
<td>93</td>
<td>7</td>
<td>79</td>
<td>18</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>-</td>
<td>1G</td>
<td>-CYA</td>
<td>27</td>
<td>22</td>
<td>92</td>
<td>8</td>
<td>96</td>
<td>0</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td>1G</td>
<td>1G</td>
<td>-CYA</td>
<td>12</td>
<td>33</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

1 Despite the apparently good control of GvHD and rejection, there was a high proportion of deaths from infection in the CAMPATH-1G/CAMPATH-1M group. Many of these patients had received additional chemotherapy with thiopeta, as well as conventional cyclo/TBI.
Table 7. HLA-matched sibling PBSC transplants: all diseases (malignant and non-malignant)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Marrow (in vitro)</th>
<th>Total cases n</th>
<th>500 day neut</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (in vivo)</td>
<td>CYA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1M</td>
<td>+CYA</td>
<td>19</td>
<td>17</td>
<td>84</td>
<td>16</td>
<td>94</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>1G</td>
<td>+CYA</td>
<td>25</td>
<td>13</td>
<td>100</td>
<td>0</td>
<td>91</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1G</td>
<td>-CYA</td>
<td>62</td>
<td>13</td>
<td>97</td>
<td>3</td>
<td>90</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>1H</td>
<td>+CYA</td>
<td>29</td>
<td>13</td>
<td>100</td>
<td>0</td>
<td>89</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>1H</td>
<td>-CYA</td>
<td>161</td>
<td>13</td>
<td>99</td>
<td>1</td>
<td>92</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>1G (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>18</td>
<td>14</td>
<td>88</td>
<td>12</td>
<td>83</td>
<td>0</td>
</tr>
<tr>
<td>1H (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>58</td>
<td>14</td>
<td>98</td>
<td>2</td>
<td>90</td>
<td>6</td>
</tr>
</tbody>
</table>
Data for Protocol 03 were analyzed in 1999 and the results published together with technical details of the transplants [9]. They were updated for this meeting (Table 7). Recovery of CD34+ cells following the in vitro incubation with CAMPATH-1G or CAMPATH-1H was close to 100%. Comparison of CAMPATH-1G and CAMPATH-1H shows some differences, which seemed to be clinically very apparent, but it is not clear whether they are statistically significant. There was more chronic GvHD using CAMPATH-1H without CYA. Although it was reported as limited, teams at Cape Town and Ulm found it to be a significant clinical issue. Transplant-related mortality was a little higher (not significant) with CAMPATH-1H and there were more deaths from infections. However, these problems seemed to be reduced with the use of post-transplant CYA. Overall, the results of PBSC transplants did not seem as good as for BM (compare Tables 3 and 7).

Transplant-related mortality was higher for PBSC, despite the favorable characteristics of these transplants: a high proportion of good-risk patients, rapid engraftment and very little graft rejection or GvHD (Figure 3). However, comparison of PBSC and BM over all protocols in the multivariate analysis showed reduced transplant-related mortality for the blood stem cells (see later).

In Protocol 07 (CAMPATH-1G/1H pre-transplant) only 19 patients received CAMPATH-1G so it was hard to compare the outcome with CAMPATH-1H (38 patients) (Table 7). Nearly all received post-transplant CYA. With CAMPATH-1H there was a very low rate of graft rejection or GvHD. Transplant-related mortality appeared high at 2 years (36 ± 27%) but few patients had been followed long enough for a reliable estimate. Many of these were poor-risk patients who received reduced intensity conditioning because they were deemed to be unsuitable for a standard transplant.

Unrelated donor transplants
As a general rule, adults have been treated according to Protocols 06 or 07, whereas children have been treated according to Protocol 02 (Tables 8–10). Good results were obtained with Protocol 02 so long as post-transplant CYA was given. However, this has been discontinued due to difficulties with the supply of rat antibodies.

Comparison of CAMPATH-1H and CAMPATH-1G using in vitro (Protocols 06, 07) showed a significant advantage for CAMPATH-1H in terms of rejection, transplant-related mortality and leukemia-free survival. Whether this is due to the antibody itself, or some other concurrent improvement in the transplant procedures, is impossible to tell. For example, the Hammersmith Hospital added an extra fraction of TBI at about the same time as introducing CAMPATH-1H. The results indicate that the humanized antibody is no worse in this setting, although there was more Grade II acute GvHD in patients treated on Protocol 06. However, at the meeting the Hammersmith team raised a note of caution, reporting a high incidence of relapse in the CML patients who received CAMPATH-1H. Since these relapses were mostly diagnosed by molecular or cytogenetic techniques, and were often successfully treated by donor lymphocyte infusions, they do not appear in the database.

The results with CAMPATH-1H pre-transplant (Protocol 07) were at least as good, if not better than CAMPATH-1H given pre- and post-transplant (Protocol 06). Engraftment was faster, rejection and GvHD were very low, and the 1-year survival data are excellent so far. The humanized antibody persists in the patient for at least 5 days, and is probably sufficient to deplete any donor T cells. Avoiding post-transplant antibody might be beneficial by favoring more rapid reconstitution of lymphocytes and other cells.

Conclusions from univariate analyses

- In general, CAMPATH-1H is at least as good as CAMPATH-1G, though there is more chronic GvHD (in the absence of CYA).
Table 8. Unrelated donor bone marrow transplants: all diseases (malignant and non-malignant)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patient</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases n</th>
<th>500 neut day</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in vivo</td>
<td>in vitro</td>
<td></td>
<td></td>
<td></td>
<td>Yes / No</td>
<td>0/1 2 3/4 O M S</td>
<td></td>
<td></td>
<td>Remit</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>+CYA</td>
<td>399</td>
<td>16</td>
<td>85 15</td>
<td>78 16 6</td>
<td>84 10 6 22</td>
<td></td>
<td></td>
<td>70 ± 4</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>+CYA</td>
<td>97</td>
<td>18</td>
<td>84 16</td>
<td>65 22 13</td>
<td>59 39 2 26</td>
<td></td>
<td></td>
<td>57 ± 10</td>
</tr>
<tr>
<td>1G/H</td>
<td>1G/H</td>
<td>+CYA</td>
<td>78</td>
<td>22</td>
<td>78 22</td>
<td>73 18 9</td>
<td>81 12 7 32</td>
<td></td>
<td></td>
<td>48 ± 9</td>
</tr>
<tr>
<td>1G (pre/post)</td>
<td>-</td>
<td>+CYA</td>
<td>535</td>
<td>22</td>
<td>82 18</td>
<td>59 23 17</td>
<td>61 28 11 36</td>
<td></td>
<td></td>
<td>68 ± 3</td>
</tr>
<tr>
<td>1H (pre/post)</td>
<td>-</td>
<td>+CYA</td>
<td>86</td>
<td>27</td>
<td>94 6</td>
<td>38 51 11</td>
<td>58 31 12 20</td>
<td></td>
<td></td>
<td>77 ± 8</td>
</tr>
<tr>
<td>1G (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>144</td>
<td>17</td>
<td>96 4</td>
<td>52 25 23</td>
<td>82 11 7 27</td>
<td></td>
<td></td>
<td>64 ± 8</td>
</tr>
<tr>
<td>1H (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>46</td>
<td>18</td>
<td>98 2</td>
<td>66 24 10</td>
<td>82 15 3 11</td>
<td></td>
<td></td>
<td>Follow-up too short</td>
</tr>
</tbody>
</table>

Table 9. Unrelated donor bone marrow transplants: acute leukemia in first remission (ALL-CR1 and AML-CR1)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Patient</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases n</th>
<th>500 neut day</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in vivo</td>
<td>in vitro</td>
<td></td>
<td></td>
<td></td>
<td>Yes / No</td>
<td>0/1 2 3/4 O M S</td>
<td></td>
<td></td>
<td>Remit</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>+CYA</td>
<td>54</td>
<td>16</td>
<td>87 13</td>
<td>86 9 5</td>
<td>92 3 5 19</td>
<td></td>
<td></td>
<td>61 ± 9</td>
</tr>
<tr>
<td>1G (pre/post)</td>
<td>-</td>
<td>+CYA</td>
<td>28</td>
<td>22</td>
<td>96 4</td>
<td>72 16 12</td>
<td>71 24 5 14</td>
<td></td>
<td></td>
<td>68 ± 13</td>
</tr>
</tbody>
</table>
Table 10. Unrelated donor bone marrow transplants: chronic myeloid leukemia in first chronic phase (CML-CP1)

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Marrow</th>
<th>CYA?</th>
<th>Total cases</th>
<th>500 neut day</th>
<th>Sustained graft (%)</th>
<th>Acute GvHD (%)</th>
<th>Chronic GvHD (%)</th>
<th>Bad %</th>
<th>Actuarial at 2 years (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>0/1</td>
<td>2</td>
<td>3/4</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>+CYA</td>
<td>39</td>
<td>17</td>
<td>72</td>
<td>28</td>
<td>59</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>1G</td>
<td>1M</td>
<td>-CYA</td>
<td>29</td>
<td>20</td>
<td>76</td>
<td>24</td>
<td>57</td>
<td>33</td>
<td>10</td>
</tr>
<tr>
<td>1G (pre/post)</td>
<td>-</td>
<td>+CYA</td>
<td>201</td>
<td>25</td>
<td>82</td>
<td>18</td>
<td>57</td>
<td>27</td>
<td>16</td>
</tr>
<tr>
<td>1H (pre/post)</td>
<td>-</td>
<td>+CYA</td>
<td>44</td>
<td>29</td>
<td>95</td>
<td>5</td>
<td>30</td>
<td>65</td>
<td>5</td>
</tr>
<tr>
<td>1G (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>26</td>
<td>20</td>
<td>88</td>
<td>12</td>
<td>32</td>
<td>36</td>
<td>32</td>
</tr>
<tr>
<td>1H (pre)</td>
<td>-</td>
<td>+CYA</td>
<td>17</td>
<td>21</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>38</td>
<td>12</td>
</tr>
</tbody>
</table>
The best practical protocols appear to be CAMPATH-1H in the bag (02) or CAMPATH-1H pre-transplant (07).
- Deaths from infection are significant in some settings — notably PBSC transplants.
- Addition of post-transplant CYA always seemed to improve the outcome.

**Results — multivariate analysis**

Ratios are expressed in this table.

Almost all factors analyzed appeared to significantly affect the rate of engraftment (Table 11). The dominant ones were: faster with high cell dose ($p < 10^{-6}$), faster with PBSC rather than bone marrow ($p < 10^{-9}$, even allowing for effect of cell dose) and faster with post-transplant cyclosporin ($p < 10^{-7}$).

**Transplant-related mortality (TRM; all patients):**

The most significant factors influencing TRM were match (less was bad), age (older was bad), and disease status at time of transplant (more advanced was bad) (all $p < 10^{-9}$) (Table 12). In addition, higher total dose of TBI was good ($p < 10^{-5}$), CAMPATH-1M alone (Protocol 01) was bad ($p < 10^{-5}$), CAMPATH-1H was good ($p < 10^{-4}$), male donor was good ($p < 0.001$), MTX

### Table 11. Multivariate analysis of time to engraftment (days to reach $0.5 \times 10^9$ neutrophils/L) (all patients)

<table>
<thead>
<tr>
<th>Good factors</th>
<th>$\beta$</th>
<th>Risk factor</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>High cell dose</td>
<td>0.17</td>
<td>1.33</td>
<td>$&lt; 10^{-9}$</td>
</tr>
<tr>
<td>PBSC rather than BM</td>
<td>0.99</td>
<td>1.15</td>
<td>$&lt; 10^{-9}$</td>
</tr>
<tr>
<td>CYA post-transplant</td>
<td>0.20</td>
<td>1.14</td>
<td>$&lt; 10^{-7}$</td>
</tr>
<tr>
<td>Higher dose of TBI</td>
<td>0.04</td>
<td>1.06</td>
<td>$&lt; 10^{-3}$</td>
</tr>
<tr>
<td>No MTX post-transplant</td>
<td>0.17</td>
<td>1.05</td>
<td>$&lt; 10^{-5}$</td>
</tr>
<tr>
<td>Male donor</td>
<td>0.11</td>
<td>1.06</td>
<td>$&lt; 10^{-2}$</td>
</tr>
<tr>
<td>Female patient</td>
<td>0.15</td>
<td>1.09</td>
<td>$&lt; 10^{-2}$</td>
</tr>
<tr>
<td>Non-TBI conditioning regimen</td>
<td>0.18</td>
<td>1.04</td>
<td>$&lt; 10^{-5}$</td>
</tr>
<tr>
<td>CAMPATH-1H</td>
<td>0.14</td>
<td>1.02</td>
<td>$&lt; 10^{-2}$</td>
</tr>
</tbody>
</table>

### Table 12. Multivariate analysis of transplant related mortality (all patients)

<table>
<thead>
<tr>
<th>Good factors</th>
<th>$\beta$</th>
<th>Risk factor</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good match</td>
<td>0.27</td>
<td>1.77</td>
<td>$&lt; 10^{-9}$</td>
</tr>
<tr>
<td>Younger than 30 years</td>
<td>0.14</td>
<td>1.36</td>
<td>$&lt; 10^{-9}$</td>
</tr>
<tr>
<td>Transplanted in disease remission</td>
<td>0.07</td>
<td>1.17</td>
<td>$&lt; 10^{-9}$</td>
</tr>
<tr>
<td>Higher dose of TBI</td>
<td>0.07</td>
<td>1.14</td>
<td>$&lt; 10^{-5}$</td>
</tr>
<tr>
<td>Not CAMPATH-1M alone</td>
<td>0.24</td>
<td>1.12</td>
<td>$&lt; 10^{-5}$</td>
</tr>
<tr>
<td>Male donor</td>
<td>0.19</td>
<td>1.11</td>
<td>$&lt; 10^{-3}$</td>
</tr>
<tr>
<td>CAMPATH-1H</td>
<td>0.38</td>
<td>1.05</td>
<td>$&lt; 10^{-4}$</td>
</tr>
<tr>
<td>CAMPATH-1M</td>
<td>0.13</td>
<td>1.08</td>
<td>$&lt; 10^{-2}$</td>
</tr>
<tr>
<td>No MTX post-transplant</td>
<td>0.18</td>
<td>1.05</td>
<td>$&lt; 10^{-3}$</td>
</tr>
<tr>
<td>CYA post-transplant</td>
<td>0.10</td>
<td>1.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Male patient</td>
<td>0.09</td>
<td>1.05</td>
<td>0.04</td>
</tr>
<tr>
<td>PBSC rather than BM</td>
<td>0.19</td>
<td>1.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>
was bad \((p < 0.001)\), CAMPATH-1G was good \((p < 0.01)\), male patient may be good \((p = 0.04)\), CYA may be good \((p = 0.04)\) and blood stem cells may be good \((p = 0.02)\).

Note that, because total dose of TBI was confounded with number of fractions and dose rate, it is not possible from these data to reach any definitive conclusions about the merits of particular radiation regimens.

**Relapse (acute leukemia)**

The analysis of relapse was limited to 1717 patients with acute leukemia (Table 13). The most significant factor was status at time of transplant \((p < 10^{-6})\). Apart from this, the only factors to emerge as significant were: a higher dose of TBI may be good \((p < 0.02)\), a female donor may be good \((p < 0.02)\) and a good match may be good \((p < 0.05)\). T-cell depletion with any antibody or T-cell add-back had no significant effect on relapse in this subclass of patients.

**Leukemia-free survival (acute leukemia)**

Significant factors were status at time of transplant (more advanced was bad) \((p < 10^{-6})\), match (poor was bad) \((p < 0.001)\), age (older was bad) \((p < 0.01)\) (Table 14). Of the factors that can be controlled, higher dose of TBI was good \((p < 0.001)\), MTX was bad \((p < 0.01)\), CAMPATH-1H may be good \((p < 0.02)\).

In summary, the use of either CAMPATH-1G and CAMPATH-1H was associated with better outcomes in these analyses, especially in reduced TRM, and no obvious increase in relapse resulting in improved leukemia-free survival. An example of a 'corrected' actuarial analysis of transplant-related mortality is shown in Figure 4. This simultaneously illustrates the benefits of better donor-recipient matching and of using CAMPATH-1H in the protocol, whilst allowing for the influence of the other significant covariates.

**Results — individual centers**

Representatives from several of the principal transplant centers presented and discussed their individual results:

University Hospital, Cape Town, South Africa

Nicolas Novitsky summarized the experience using Protocol 03 (CAMPATH-1G or CAMPATH-1H 'in the bag'). A total of 79 BM transplants and 66 PBSC transplants were carried out (all Protocol 03). CAMPATH-1G was used in 106 and CAMPATH-1H in 39 cases (all PBSC). Based on the mononuclear cell number in the graft and using approx 0.05 mg antibody per 10⁶ cells, the

| Table 13. Multivariate analysis of leukemia relapse (acute leukemia only) |
| Good factors | β | Risk factor | P value |
| Transplanted in disease remission | 0.20 | 1.58 | < 10^{-9} |
| Higher dose of TBI | 0.06 | 1.14 | 0.02 |
| Female donor | 0.22 | 1.13 | 0.02 |
| Good match | 0.07 | 1.14 | 0.05 |

| Table 14. Multivariate analysis of leukemia-free survival (acute leukemia only) |
| Good factors | β | Risk factor | P value |
| Transplanted in disease remission | 0.19 | 1.54 | < 10^{-9} |
| Good match | 0.10 | 1.21 | < 10^{-3} |
| Higher dose of TBI | 0.07 | 1.15 | < 10^{-3} |
| Younger than 30 years | 0.05 | 1.10 | < 10^{-2} |
| No methotrexate post-transplant | 0.19 | 1.05 | < 10^{-2} |
| CAMPATH-1H | 0.26 | 1.03 | 0.02 |
Figure 4. Multivariate analysis of transplant-related mortality (all patients). The risk of dying from causes other than relapse is plotted, taking into account all other significant factors identified in the multivariate analysis. Four groups are illustrated here: less well matched donors (unrelated or mismatched family members) and better matched donors (HLA-identical siblings or HLA-identical other family members). Each is subdivided into patients who received CAMPATH-1H for GvHD prophylaxis and patients who received other CAMPATH antibodies.

A typical dose of CAMPATH-1H was 20–40 mg. Usually no post-transplant immunosuppression was given, but following the occurrence of chronic GvHD in the CAMPATH-1H group, prednisone (or occasionally CYA) was administered up till Day 90. Due to the referral patterns to hospital, CYA therapy was not always practical, as it required frequent visits by patients for monitoring. To date, in the 20 patients treated with prophylactic prednisone post-transplant, mild chronic GvHD was still seen, but delayed. This complication was not seen in subjects treated with CYA. Patients are now routinely given septrin and penicillin.

Individuals treated with CAMPATH-1G showed a rather poorer outcome for PBSC compared with BM, although there were no significant differences in early (< 30 day) or late mortality between CAMPATH-1G and CAMPATH-1H. The main observations on moving to CAMPATH-1H were a higher rate of CMV reactivation (not fatal, but sometimes leading to other complications, e.g. graft failure and sepsis), and a higher rate of chronic GvHD (42%). Although chronic GvHD was generally responsive to steroid treatment, this therapy could lead to infection complications. Some patients in the CAMPATH-1H group who had recovered hematopoiesis developed overwhelming sepsis with meningococcal or staphylococcal pneumoniae, which had not been observed previously.

Roel Willemeze and Grant Prentice remarked that a high rate of CMV reactivation was to be expected and possibly may be seen earlier after PBSC transplants compared with BM transplants. High dose acyclovir is used as CMV prophylaxis at the Royal Free Hospital, with twice-weekly monitoring by PCR and pre-emptive therapy given following two consecutive positive PCR results. Deaths from CMV disease were rare, but nevertheless viral reactivation remains a substantial issue.

Recovery of lymphocyte subsets post-transplant was studied at Cape Town, and the outcome following allologeneic BMT, autologous and allogeneic PBSC compared. Some of this work has been published [10]. The main findings were as follows:

- Despite an increase in colony-forming units granulocyte–macrophage (CFU-GM), there was no significant difference in the reconstitution of lymphoid subsets between BM and PBSC transplants.

- Although memory T cells (CD2*CD45RA*) were expanded, the naive population remained low during 12 months follow-up — this was most obvious in the CD4*CD45RA+ subgroup. Adoptive immunotherapy is unlikely to correct this and ways to improve thymic recovery need to be reviewed.

- CD95 (Fas — normally characteristic of activated cells) was over-expressed during the follow-up period by all transplant groups.

University Hospital, Ulm, Germany

Donald Bunjes described the results obtained between 1989 and 1997 for 43 patients with AML in first remission transplanted according to Protocol 02 (CAMPATH-1G
and CAMPATH-1M) following standard conditioning with Cyclo/TBI. Median follow-up is now 7 years. There was no rejection and the median time to reach 0.5 × 10⁹ neutrophils/L was 23 days. There was no acute GVHD > Grade II and only 9% chronic GVHD. Only four patients had any immunosuppression post-transplant and the long-term DFS is 66%.

Since then, 48 patients have been treated according to Protocol 03, all with CAMPATH-1H (20 mg). This cohort was older, had higher-risk disease and included about 20% non-HLA-identical related donors. Thiopeta was added to the conditioning regimen for some of the patients. No post-transplant CYA was given. Again, there was no graft failure. Engraftment (0.5 × 10⁹ neutrophils/L) occurred by Day 11. In contrast with the previous results, there was a 60% incidence of acute GVHD (all < Grade 2) and 30% chronic GVHD. Although the GVHD was not severe, it did require treatment, which Donald did not find acceptable. The transplant-related mortality in this cohort was 38%, about double that of the previous trial — though the patients were worse risk. CMV reactivation occurred in about 90% of patients at risk (i.e. seropositive before transplant) and one died of CMV pneumonitis. CMV reactivation and its subsequent treatment was likely to predispose to other infectious complications. Overall, immune reconstitution was seen as the biggest outstanding problem.

Two suggestions were proposed to modulate the incidence of GVHD:

- Add CYA post-transplant;
- Add in vivo antibody pre-transplant.

Stephen Mackinnon commented that at the Memorial Sloan Kettering Institute, the combination of thiopeta and CYA had proved to be very toxic. Donald Bunjes replied that since January 2000, CYA has been introduced at Ulm and thiopeta dropped. Nicolas Novitzky suggested that the relatively high incidence of chronic GVHD with little acute GVHD may be related to the relatively high proportion of ‘TH2 type’ (i.e. IL6⁺, IL2⁻) CD8⁺ cells in PBSC compared with BM. Donald Bunjes answered that a relatively high number of cases of autoimmune disease (cytopenias and thyroiditis) had been seen following PBSC transplant, compared with BM, perhaps consistent with this hypothesis.

University Hospital, Leiden, The Netherlands
Roel Willemsje presented the data on PBSC transplants at Leiden using Protocol 03. So far, none of the patients has received post-transplant CYA. To date, 33 patients had received stem cells treated with CAMPATH-1G and 24 with CAMPATH-1H. The incidence of acute GVHD > Grade I was 11% (CAMPATH-1G) or 29% (CAMPATH-1H). The total incidence of chronic GVHD was 17% (CAMPATH-1G) or 34% (CAMPATH-1H). Although there was more GVHD in the CAMPATH-1H group there was not yet a significant difference in transplant-related mortality. Possibly the results would be improved by introduction of post-transplant CYA, but it was correctly pointed out that this had not previously been advocated since one of the principal benefits of T-cell depletion had been the avoidance of post-transplant immunosuppression.

Royal Free Hospital, London, UK
Grant Prentice described the results using CAMPATH-1H both in vivo pre-transplant and in the bag (Protocol 04). A total of 28 patients, aged up to 57 years (median 36 years), suffering from various diseases (including high-risk) were transplanted from various donors, including 21 alternative donors, mostly unrelated and many partly HLA-mismatched. Sixteen received BM treated with 10 mg CAMPATH-1H ‘in the bag’ and 12 received PBSC treated with 20 mg CAMPATH-1H. The conditioning regimen originally contained TLI and busulfan, but more recently a protocol of fludarabine, cyclophosphamide, TBI and CAMPATH-1H (5 days) has been used. HLA-matched patients (whether related or not) did not receive post-transplant immunosuppression; others had CYA.

Two patients transplanted from unrelated donors did not engraft; however lab tests on the pre-transplant BM samples showed viability but no growth in CFU-GM culture. There was no acute GVHD > Grade II, six had limited chronic GVHD and one extensive chronic GVHD. As observed by other centers, there was a high degree of reactivation of CMV, but CMV disease was prevented by monitoring and pre-emptive therapy, as described earlier. One patient who suffered Grade II acute GVHD later died of adenovirus infection. CD4⁺ T cells had risen to > 200/µL by about 9 months post-transplant — this was not delayed compared with previous patients at the Royal Free Hospital. T-cell recovery was a little faster for
sibling donors compared with unrelated donors. In conclusion, CAMPATH-1H was well tolerated, there was little GvHD, but a high degree of CMV reactivation.

Heartlands Hospital, Birmingham, UK

Donald Milligan reported the results for two groups of patients — the first received 'mini' transplants using fludarabine, melphalan and CAMPATH-1H for conditioning with T-replete stem cells (Protocol 07, 14 patients). The second group received conventional conditioning with cyclophosphamide and TBI (sometimes with etoposide as well) and PBSC treated with CAMPATH-1H (Protocol 03, 14 patients). All of the patients received post-transplant CYA. There was little or no graft failure or GvHD in both groups, but an issue of significant concern in the mini-transplant group was the incidence of viral infections, particularly CMV (8/9 patients at risk, one died), adenovirus (7/14 patients at risk, one died) and polyomavirus (6/14 patients at risk). The frequency of other viral infections, including EBV and HSV, were similar and low in both groups. In total, despite the small numbers of patients, there were significantly more virus infections in the mini-transplant group. Lymphocyte counts, including CD4 T cells and cytotoxic T-cell responses were lower in the mini-transplant group than in the conventional-transplant group. Could these differences be due to the different total dose of CAMPATH-1H, or some other factor, such as the use of fludarabine, or differences in the patient population? A proposed strategy is to use just 10 mg of CAMPATH-1H 'in the bag' instead of in vivo with the mini conditioning regimen.

University College Hospital, London, UK

Stephen Mackinnon presented data on mini-transplants updated since a recent publication [11]. The basic protocol consisted of 5 × 20 mg CAMPATH-1H from Day −8 to Day −4, fludarabine from Day −7 to Day −3, melphalan on Day −2 and CYA post-transplant, originally for 6 months, but now for 1–3 months. CAMPATH-1H was included to prevent rejection, but it also resulted in a low incidence of GvHD, which allowed the CYA to be reduced. This protocol has been used with a variety of high-risk patients unsuitable for conventional transplants owing to age or a previous transplant. HLA-identical siblings (mostly PBSC) and unrelated donors (mostly BM) were included. Of 64 patients analyzed, 14 had been transplanted in relapse or with refractory disease and 30 were second transplants. One patient died before engraftment, one suffered graft rejection and the other 62 all engrafted. There were typically 5–7 days of neutropenia. Chimerism was measured by mini-satellite analysis and it was found that about 50% were fully donor type at first analysis. About 25% were mixed chimeras in all lineages and the remaining 25% were mixed chimeras for T cells and full in others.

Only five patients suffered any acute GvHD and none was > Grade II. There was almost no chronic GvHD, even when CYA was stopped early. Transplant-related mortality at 1 year was 17%. Engraftment was good even among the 20 patients transplanted from unrelated donors, including eight who were partly HLA-mismatched. However, the follow-up was an average < 1 year and all patients were still at risk of relapse. They were not routinely given donor lymphocytes, but these were started at 6 months if there was mixed chimerism and evidence of residual disease. Lymphocytes were given cautiously, starting with 10⁴ per kg and even this has produced some GvHD (other groups have used 10 times more). It is estimated that a safe dose for T cells is of the order of 10⁵/kg at the time of transplant, but may rise to 10⁶/kg after several years. Older patients such as these may not tolerate GvHD.

As in other studies, immune reconstitution was slow, with also a high frequency of CMV reactivation, causing a logistical problem with the need for frequent testing and pre-emptive treatments. One possibility might be the adoptive transfer of CMV-specific donor T cells. Following infusion of about 10⁷/kg CMV-specific donor T cells in 2–3 weeks there was a huge proliferation, despite the continued administration of CYA, and these cells could be followed in vivo by using CMV-specific HLA-peptide tetramers.

The possibility of disordered immune reconstitution was discussed. Donald Milligan described one patient who died from severe autoimmune hemolytic anemia, and one other who died from tuberculosis in the spleen. Other published mini-transplant protocols have mentioned similar problems. However, Stephen Mackinnon pointed out that the transplant-related mortality in the patients who received CAMPATH-1H is still lower than for other protocols, for example in a European study (including the UK data as about one-third of the dataset), the transplant-related mortality at 100 days was 11% and
at 1 year was 30%. GvHD was a common complication in the European study, perhaps partly because donor lymphocytes were being given too early.

Herman Waldmann asked whether there was any evidence for an adjuvant effect of CAMPATH-1H in preventing relapse in lymphoid malignancies. The patients were too heterogeneous and the data too early to answer this question, but at 3 months post-transplant most were in complete or partial remission (having generally been transplanted in relapse or with resistant disease), suggesting that the regimen gives good disease control, at least initially.

Discussion
CAMPATH-1G and CAMPATH-1H appear to be generally similar in activity for depleting T lymphocytes to prevent GvHD and rejection. However, differences were reported. On the one hand, there was a significant advantage in leukemia-free survival associated with the use of CAMPATH-1H in unrelated donor transplants (Protocols 06, 07). On the other, there was an increased incidence of chronic GvHD and possibly poorer transplant-related mortality in PBSC transplants where CAMPATH-1H was used to treat the stem-cell infusion (Protocol 03). All of these comparisons are with retrospective historical groups and there have been no prospective randomized trials, it is therefore impossible to tell for certain whether the differences are related to the change in the Ab, or some other factors. One factor is that the humanized antibody could have a longer half-life in vivo and this might have an impact on the pattern of lymphocyte regeneration in the weeks following a transplant. Some preliminary studies in unrelated donor transplants suggest that this might be the case [8].

It is undisputed that T-cell depletion is the most effective way to prevent GvHD, but it is difficult to balance this benefit against the possible loss of graft versus tumor activity and delay in immune reconstitution. There is no doubt that the numbers of T cells in the blood, particularly CD4+ cells, recover more slowly after a T-cell depleted transplant. However, it is less clear whether there is a corresponding functional impairment in immunity. Many lines of evidence are suggesting that the initial expansion derives from donor memory cells, which may lead to a relatively restricted oligoclonal repertoire. Emergence of new naïve T cells from the thymus is very slow, but there is no reason to suppose it will be diminished by T-cell depletion of the donor graft — on the contrary, it has been suggested that the relative lack of peripheral lymphocytes may facilitate that process.

An unexpected finding was the significant benefit of post-transplant CYA in virtually every protocol. It was not remarkable that this might further reduce the incidence of GvHD and rejection and thus reduce transplant-related mortality, but the effect appeared to persist even when these factors were allowed for. An important benefit of protocols containing CYA appeared to be in the reduction of deaths from infections during the period from approx. 6 months to 18 months post-transplant (during a time when most patients would already have discontinued the drug). There was much discussion at the meeting about the possible explanations. For example, CYA might prevent sub-clinical GvHD, which would otherwise compromise the fragile immune system. Alternatively, perhaps the patients who received the immunosuppressive CYA were monitored more effectively, or were given better prophylactic antibiotic regimens. Maybe cyclosporin has a counter-intuitive favorable influence on immune reconstitution after T-cell depleted transplants. Unfortunately, we do not have the data to address these questions.

At the end of all the analysis, Protocols 03 and 07 were commended for further study, with particular emphasis on elucidating the role of post-transplant CYA in this context. The group will continue to follow up the existing cohorts of patients for long-term effects and outcomes. However, it seems that with the likely wider availability of CAMPATH-1H, it is time for prospective randomized trials to be carried out to establish the real utility of this treatment in stem-cell transplantation, and to compare it directly with more conventional approaches.

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We thank everyone who contributed to this meeting, including not only the speakers and discussants, but also the many members of transplant teams who contributed data for analysis as well as the organizations that have provided funding over the years for supply of CAMPATH antibodies: UK Medical Research Council, The Wellcome Foundation Ltd, Leukosite Inc, Millennium Pharmaceuticals Inc., the Kay Kendall Foundation and the EP Abraham’s Trust.
References


Blood concentrations of alemtuzumab and antilglobulin responses in patients with chronic lymphocytic leukemia following intravenous or subcutaneous routes of administration

Geoff Hale, Peppy Rebelo, Lee R. Brettman, Chris Fegan, Ben Kennedy, Eva Kiniby, Mike Leach, Jeannette Lundin, Håkan Mellstedt, Paul Moreton, Andy C. Rawstron, Herman Waldmann, Anders Österborg, and Peter Hillmen

Alemtuzumab is a humanized anti-CD52 antibody licensed for refractory B-cell chronic lymphocytic leukemia (B-CLL), when given intravenously at 30 mg thrice weekly. However, the intravenous route is associated with infusion-related reactions and is inconvenient. We measured blood concentrations in 30 relapsed patients treated with intravenous alemtuzumab and in 20 patients from a previously untreated group who received similar doses subcutaneously. Highest trough samples in the intravenous group were less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The cumulative dose required to reach 1.0 μg/mL was 13 mg to 316 mg (mean 90 mg). Higher blood concentrations correlated with the achievement of better clinical responses and minimal residual disease. The highest measured concentrations in the subcutaneous group were similar (0.6 μg/mL to 24.8 μg/mL, mean 5.4 μg/mL). However, the cumulative dose to reach 1.0 μg/mL was higher: 146 mg to 1106 mg (mean 551 mg). No antilglobulin responses were detected in 30 patients given intravenous alemtuzumab whereas 2 of 32 patients given subcutaneous alemtuzumab made substantial anti-idiotypic responses. Thus, subcutaneous alemtuzumab achieved concentrations similar to those for intravenous alemtuzumab, although with slightly higher cumulative doses. Subcutaneous alemtuzumab is more convenient and better tolerated but may be associated with some patients forming anti-alemtuzumab antibodies, particularly those patients who were previously untreated. (Blood. 2004;104:948-955)

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Introduction

Alemtuzumab (CAMPATH-1H, Campath; ILEX Pharmaceuticals, San Antonio, TX) is a humanized immunglobulin G1 (IgG1) antibody that recognizes the CD52 antigen, a lipid-anchored glycoprotein, on lymphocytes.1,2 Alemtuzumab is exceptionally lympholytic and has been tested as an immunosuppressive agent in transplantation and autoimmune diseases. It is active against a range of lymphoid malignancies and in 2001 was approved for the treatment of B-cell chronic lymphocytic leukemia (B-CLL) in patients who have been treated with alkylating agents and who failed fludarabine therapy. This was based largely on a trial in 93 patients, most of whom had advanced disease with an extremely poor prognosis.4 After a short dose escalation, alemtuzumab was given intravenously at 30 mg thrice weekly for 4 to 12 weeks. This gave an overall response rate (complete remission [CR] + partial remission [PR]) of 33%. Other trials in chemoresistant CLL reported comparable response rates of 42% and 29% respectively.5,6

The dose regimen was developed empirically without the benefit of detailed pharmacodynamic or pharmacokinetic studies. Alemtuzumab often causes substantial first-dose reactions, and although the reactions can now be minimized by appropriate premedication, they are the reason why an initially escalating dose was followed by frequent small doses. This regimen is not the most convenient for patients or physicians. Therefore, the subcutaneous route has been studied in an attempt to reduce side effects and make the treatment more manageable.7,8 The different routes of administration can be compared by analysis of the biodistribution of antibody, on the principle that treatments that give comparable blood concentrations should produce similar clinical effects. Some information is available about the pharmacokinetics of intravenous alemtuzumab in patients undergoing stem cell transplantation who have no substantial leukemic burden.9,10 The terminal half-life was estimated to be approximately 15 to 20 days. However, in patients with advanced CLL, tumor cells could absorb antibody from the circulation.

The humanization of alemtuzumab reduced the risk of antilglobulin responses.11 However, the antigen binding sites are still potentially immunogenic. The ability to provoke a response depends on the target antigen, the immune status of the patient, the use of immunosuppressive drugs, and other factors, perhaps including the route of administration. There is a suggestion that subcutaneous administration might be more immunogenic than...
intravenous administration in patients with rheumatoid arthritis, though it is well known that such patients are particularly able to make anti-lymphocytes.

Here we measured blood concentrations of alemtuzumab and anti-lymphocyte antibodies against it during 2 different clinical trials. In the first, alemtuzumab was administered intravenously to patients who had previously failed alkylating agents and fludarabine as in the licensed indication. In the second, alemtuzumab was administered subcutaneously to patients who required therapy but had received no prior treatment. We compared the blood concentrations and anti-lymphocyte antibodies and investigated the possible relationship between alemtuzumab concentrations and clinical responses.

Study design: A second study

An open-label phase 2 trial was conducted at 4 clinics in the United Kingdom and in Stockholm, Sweden. The primary objective was to assess the efficacy of alemtuzumab administered subcutaneously. Patients were eligible if they had a diagnosis of R-CLL, were 18 years of age or older, had a WHO performance status of 1 or less, life expectancy at least 12 weeks, required treatment and had not been treated previously. Other details were similar to the first study except the treatment regimen. Alemtuzumab was administered from the Therapeutic Antibody Centre, Oxford, United Kingdom (patients 1-29) or from MedPartners (patients 30-41). The 2 preparations were similar although the formulations were slightly different and the antibody from the Therapeutic Antibody Centre was stored frozen. On days 1, 3, 5, 7, and 9, alemtuzumab was administered subcutaneously in the thigh. If well tolerated, 10 mg was given on day 3 and 30 mg (in 2 injection sites) on day 3. In the event of thrombosis or edema, the same dose was repeated until well tolerated. After dose escalation, most patients self-administered the antibody. The 30-mg dose was given thrice weekly for up to 18 weeks. If treatment was interrupted for more than 7 days the dose was reinitiated at 3 mg or 10 mg. Therapy was stopped if patients achieved a complete remission (CR) or fulminated NCI criteria for progressive disease (PD).

Measurement of alemtuzumab in patient serum

Serum samples were collected before doses and at various times afterward and stored at -70°C until analysis by immunoassay. Test samples were incubated at 50°C for 30 minutes to inactivate complement, then incubated with HUT-78 cells at room temperature for 30 minutes. The cells were washed and resuspended in fluoroscein isothiocyanate (FITC)-labeled polyclonal anti-human IgG Fe domain (F-9512; Sigma, Poole, United Kingdom), incubated for 30 minutes, washed and fixed with formaldehyde prior to flow cytometry. Alemtuzumab concentrations were calculated from the median fluorescence intensity on a standard curve. The lower limit of quantitation was 0.5 μg/ml (allowing for 2-fold sample dilution), overall precision was µ±10%, and overall accuracy was 100%. There was no interference by normal or patient control sera and no reactivity with F(ab')2 fragments of alemtuzumab.

Measurement of anti-lymphocyte antibodies

Antibodies to alemtuzumab were measured in serum samples by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described with some modifications. Microtiter plates were coated with alemtuzumab (M&K Pharmaceuticals) and blocked with dextran (phosphate-buffered saline [PBS] containing 2% bovine serum albumin [BSA]). Standards, quality-control samples, and test samples were added, incubated for 1 hour at room temperature, and rinsed 4 times with wash buffer (PBS containing 0.05% Tween 20). Biotin-labeled alemtuzumab was added to each well, incubated for 1 hour, and rinsed as before. The assay was developed withextravidin-peroxidase (E-2886; Sigma) and ortho-phenylenediamine. Color development was measured at 450 nm for 20 minutes by Genesis II software (Thermo Life Sciences, Basingstoke, United Kingdom). The standard was Y1D1, a monoclonal anti-idiotypic specific for alemtuzumab. One U/ml of anti-lymphocyte activity is the signal equivalent to 1 mg of Y1D1 reference standard. The limit of detection (LOD) and lower limit of quantitation (LLQ) were 488 U/ml (allowing for 2-fold sample dilution), overall precision was ±10%, and overall accuracy was 100%. There were no false positives or interferers by normal or patient control sera. Mean values were obtained with serum containing rheumatoid factor or spiked with native CD20 antigen. The assay was reproducible despite variations in reagent concentrations, incubation and washing conditions, blood clotting conditions, and sample storage conditions within the specified range. Up to 3-fold enhancement of anti-lymphocyte activity was seen in samples that had been heat-treated or stored for over a month at 4°C. This might be due to aggregation, since in the absence of the complex anti-idiotypic reagent gave enhanced activity. Alemtuzumab and anti-lymphocytes interact with each other to reduce the signal in either assay if both were present in the same sample. Thus, the

**Patients, materials, and methods**

**Patients**

Clinical studies were carried out with the approval of local and, in the United Kingdom, regional ethical committees. All patients gave written informed consent prior to enrolment.

**Study design: An open-label study**

An open-label phase 2 trial was conducted at 5 centers in the United Kingdom (Aberdeen, Birmingham, Bournemouth, Glasgow, Leeds). Primary objectives included the assessment of safety and efficacy as well as measurement of pharmacokinetics. Patients were eligible if they were at least 18 years old, had confirmed B-CLL requiring treatment, had failed previous treatment with purine analogs, had a World Health Organization (WHO) performance status of 2 or less, and had creatinine and bilirubin concentrations less than twice the upper limit of normal (unless due to disease). Exclusion criteria were active infection, HIV positivity, pregnancy or lactation, history of lymphocytosis or lymphoma, less than 3 weeks since chemotherapy or less than 6 weeks since investigational therapy, central nervous system (CNS) involvement or persisting severe pancytopenia, or severe concurrent diseases, secondary malignancy, or mental disorder.

Patients were premedicated with cefazolin and chlorothalidone 30 minutes before the first infusion. Septins (sulfadiazine/trimethoprim 150 mg/160 mg twice a day, 3 times per week) and acyclovir (200 mg thrice daily) were given prior to therapy and continued until normal blood lymphocytes returned to the normal range. Allopurinol was given during the first 4 weeks of treatment. Alemtuzumab (M&K Partnership, Cambridge, MA) was diluted to 100 mL of 0.9% saline and administered intravenously over approximately 2 hours. A dose of 3 mg was given on day 1 and 5 if tolerated, a 0.5-mg dose was given on day 2 and a 160-mg dose on day 3. If severe reactions occurred, the same dose was repeated daily until it was well tolerated and then the dose was escalated. Subsequent 0.5-mg doses were given thrice weekly for up to 12 weeks, though treatment was occasionally extended if clinically indicated.

Before therapy, a full history, physical examination, and laboratory evaluation were performed, including measurement of lymphocyte, liver, and spleen size, computed tomographic (CT) scan, Rai staging, blood analysis, bone marrow aspirate, and trephine and immunophenotyping by flow cytometry. Disease parameters were reassessed and blood and bone marrow were remeasured by flow cytometry for residual CLL cells every 4 weeks. Therapy was stopped if there was no evidence of CLL or if sequential bone marrow samples showed no fall in the level of CLL. Blood samples were collected weekly to measure alemtuzumab concentrations and anti-lymphocytes. After the last dose, samples were collected daily for 4 days and then weekly for 3 weeks. This was not always possible for logistical or clinical reasons.

During follow-up, patients were assessed monthly for 6 months. Responders were assessed every 6 months thereafter until disease progression. Disease response and toxicity were graded according to the criteria of the National Cancer Institute (NCI).
antiglobulin assay measures only the concentration of free anti-
alemtuzumab antibody, that is, the excess over the levels of alemtuzumab itself. Considering that alemtuzumab concentration was never more than 26 
μg/mL, then it might interfere with measurement of antiglobulin responses 
less than about 26 000 U/ml. However, the antiglobulin levels in a patient 
who made a clear-cut response exceeded 10^6 U/ml. We report data for all 
samples measured, both during and, where available, after treatment. For 
complete assessment of antiglobulin responses, it is always preferable to 
analyze samples over an appropriate period of time after the end of 
treatment.

Measurement of minimal residual disease by flow cytometry

Residual CLL cells were quantified by sensitive 4-color flow cytometry; a 
method more sensitive than consensus primer polymerase chain reaction 
(PCR) and more practicable than sequence-specific quantitative PCR. At 
least 5 × 10^6 bone marrow leukocytes were incubated with a mixture of 
fluorescent-labeled antibodies, washed twice, and analyzed using a FACSort 
(Becton Dickinson, Oxford, United Kingdom) with CELLQuest v3.1 
software. The antibodies used were CD45/CD48 (Chemicon, Chanders 
Ford, United Kingdom)/CD19/CD3, kappa/lambda/CD19/CD3, CD20 
(Beckman Coulter, High Wycombe, United Kingdom)/CD79a (Beckman 
Coulter)/CD19/CD3, and CD20 (Beckman Coulter)/CD3/CD79b (BD bio-
sciences, Oxford, United Kingdom)/CD19/CD3 conjugated to FITC/ 
phycocerythrin (PE)/PE-Cy5/allophycocyanin (APC), respectively. Re-
agents without named manufacturer were produced in-house. Between 
30 000 and 500 000 cells were analyzed. B cells were identified by setting 
a region on CD19 versus side scatter (SSC), followed by a forward scatter 
region, and then ensuring that no CD3^+ events fell within the combined 
gate. CLL cells were discriminated from mature B cells by their stronger 
expression of CD5 and weaker expression of CD20 and CD79b, and from T 
progenitors by their weaker expression of CD38. This assay can detect 1 
CLL cell in approximately 10^6 to 10^7 leukocytes.

Results

Collection and analysis of samples

Serum samples were obtained from all 30 patients in the intrave-
nous study and 20 of the 41 patients in the subsequent study, 
equally almost from a single clinic (Karolinska Hospital). Samples were tested in duplicate and the mean concentration was 
calculated. If the mean was less than 0.5 μg/mL (LLOQ), a level of 
less than 0.5 μg/mL was recorded. If either result was more than 20 
μg/mL or a measurement was technically faulty, then the test was 
repeated, using a higher dilution if appropriate, and the repeat 
results were used.

Optimal time for collection of peak samples following 
intravenous administration

A preliminary experiment was carried out to identify the time 
following intravenous administration when the peak antibody 
concentration might be found. Samples were collected from 3 
patients at 15 minutes, 30 minutes, and 60 minutes after the end of 
one dose each week for 4 to 7 weeks. The peak sample occurred at 
15 minutes, significantly more often (21 times) than at 30 minutes 
(6 times) or 60 minutes (2 times) (P < 0.1); paired Wilcoxon signed 
ranking test. Therefore, for subsequent patients a single sample was 
collected at 15 minutes to determine the peak concentration.

Serum concentrations of alemtuzumab during 
intravenous treatment

e total of 1561 patient samples were tested. Terminal samples 
(after the last dose) were not available for some patients, and in a 
few cases only a small number of terminal samples were available.

Figure 1. Example of alemtuzumab concentrations in patient treated with 
intravenous antibody. After an initial dose escalation, 30 mg of alemtuzumab was 
administered 3 times a week for 8 weeks (O). Serum samples were taken before (X) 
and after (Y) the dose once a week. This patient made a good clinical response to 
the therapy.

All samples were tested in duplicate; the overall mean coefficient of 
variation (CV) was 10%. Many of the early samples and many 
trough samples were below the limit of quantitation (< 0.5 
μg/mL). Five samples gave anomalous results, substantially diverging 
from other results for the same patients. These could be 
explained by accidental mix-ups at the clinical centers and were 
omitted from analysis. An example of the doses and blood 
concentrations throughout the course of treatment for one patient is 
shown in Figure 1.

In contrast to patients who had received transplants, there 
were wide variations in alemtuzumab concentrations between 
different patients with CLL. The highest peak ranged from 2.8 
μg/mL to 26.4 μg/mL (mean 10.7 μg/mL). The highest trough ranged 
from less than 0.5 μg/mL to 18.3 μg/mL (mean 5.4 μg/mL). The 
cumulative dose before the trough concentration reached 1.0 
μg/mL ranged from 13 mg to 316 mg (mean 90 mg; Table 1). In 9 
of 21 evaluable patients, the highest trough concentration was 
measured just before the last dose, indicating that a steady state had 
not been reached. Total cumulative doses varied between patients, 
but did not correlate with the final antibody concentrations 
(Table 1).

Comparison of the starting lymphocyte count with the peak 
concentration following the first 30-mg infusion showed a modest 
negative correlation, that is, the higher the initial lymphocyte 
count, the lower tended to be the peak concentration of alemtu-
zumab (Figure 2).

In Figure 3 the highest trough concentration for each patient is 
pictured against the response measured by NCI criteria for 27 
patients where the clinical response was reported within 4 months 
of the end of treatment. There was a significant increase in trough 

Figure 2. Relationship between initial CLL count and alemtuzumab blood 
concentration: Intravenous study. The numbers of CLL cells in the blood directly 
before intravenous therapy are plotted against the peak concentration of alemtu-
zumab measured directly after the first 30-mg dose (O). In 8 patients the concentra-
tion was below the limit of quantitation, and these have all been plotted at an arbitrary 
level of 0.25 μg/mL (X). Pearson correlation coefficient for log (initial CLL count) 
versus log (alemtuzumab concentration) was -0.44, P < .01.
Table 1. Alemtuzumab dose and serum concentrations: intravenous study

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Cumulative dose, mg</th>
<th>Number of samples</th>
<th>Terminal samples</th>
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ND indicates not determined.

Concentrations with increasing clinical response (P <.006, Kruskal-Wallis test). There was an even more striking correlation between trough concentrations and the attainment of minimal residual disease (MRD) negativity in 7 patients (filled symbols) compared with the 17 patients who still had residual CLL cells in the bone marrow at the end of treatment as measured by 4-color flow cytometry (P < .0001, Kruskal-Wallis test).

In Figure 4 the mean trough concentration is plotted throughout the treatment for 2 groups of patients: (1) 8 responders who reached MRD less than 0.4%, and (2) 22 others. The mean trough concentration was significantly higher in the responders throughout the whole course of treatment from week 2 to week 11 (P < .02 at each time point, Kruskal-Wallis test).

Clearance of alemtuzumab following the last dose showed a similar correlation with residual disease. In patients with undetectable CLL cells, there was a single clearance phase characterized by

![Figure 3](image-url)  
**Figure 3.** Relationship between maximum trough concentrations of alemtuzumab and clinical response: Intravenous study. The highest trough concentration of alemtuzumab (which generally occurred following the penultimate or last dose) is plotted against the clinical response at the end of intravenous treatment as determined by WBC count. PD, progressive disease; SD, stable disease; PR, partial remission; CR, complete remission. A better clinical outcome was significantly associated with higher alemtuzumab concentrations (P < .006, Kruskal-Wallis test). There was an even stronger correlation between high trough concentrations (> 5 μg/mL) and good responses measured by less than 0.1% CLL cells in the bone marrow (<0.001). Three patients who died before the end of the planned treatment course are not included in this figure. Of those patients, 2 had low alemtuzumab concentrations (< 1.1 μg/mL) and progressive disease at the time of death. The third had a high anody concentration (183 μg/mL) and low levels of residual disease (0.2%). Each symbol represents a single patient; • indicates a patient with no detectable MRD; ○, a patient with detectable MRD.

![Figure 4](image-url)  
**Figure 4.** Mean trough concentrations of alemtuzumab during Intravenous therapy. The mean trough concentrations during treatment (measured 48 hours after a dose, once a week) are plotted, with standard deviations, for 8 patients who ultimately reached less than 0.4% CLL cells in the bone marrow (□) and compared with 22 patients who still had residual CLL cells at the end of treatment (●).
a relatively long half-life. In patients who still had a substantial tumor burden, most of the antibody was rapidly cleared from the blood. Patients with low levels of residual disease showed an intermediate pattern. An example of each is shown in Figure 5.

Sixteen patients contributed informative terminal samples after the last 30-mg dose. From these data the following mean pharmacokinetic parameters were calculated (ILEX Oncology, unpublished data, September 2001). The apparent steady-state volume of distribution (VSS) was 0.185 L/kg and the apparent volume of distribution during the terminal phase (VZ) was 0.252 L/kg. These are larger than for other monoclonal antibodies in humans but are consistent with the notion that alemtuzumab distributes through the plasma compartment and a substantial extracellular lymphocyte compartment. The mean terminal phase half-life (t1/2) was 6.1 days, which is consistent with previous measurements for alemtuzumab and other chimeric antibodies in humans.

Serum concentrations of alemtuzumab during subcutaneous treatment

This study was started in 1998 using alemtuzumab manufactured at the Therapeutic Antibody Centre. During June 2000, commercially produced material became available and was subsequently used. No obvious differences were observed in the clinical responses. A total of 542 patient samples were received and tested. Two were mislabeled and were not included here. The overall mean CV was 8%. Assessment of pharmacokinetics in 7 patients was inadequate due to the limited number or inappropriate timing of samples.

Patient no. 8036 (who started with alemtuzumab from the Therapeutic Antibody Centre and was switched to commercial alemtuzumab) never achieved concentrations above the LLOQ. This patient had rapidly made a strong anti-IgG1 response to the alemtuzumab, which probably neutralized all of the administered antibody (Antibodies following subcutaneous treatment). The patient never showed any clearance of lymphocytes but showed continued reactions at the injection site. Because of this, the patient was switched to intravenous administration after 6 weeks.

There were some changes from the dose regimen due to clinical reactions. There were 2 patients who received lower doses throughout (10 mg and 20 mg) and 2 (including patient no. 8036, above) who were switched to intravenous dosing. The dose escalation rate was often slower than originally planned, typically taking 1 to 2 weeks to reach 30 mg. After this, the planned dose was usually maintained but only 1 of the 21 patients analyzed received the maximum planned dose of 1573 mg. The mean total dose was 1249 mg and the lowest dose was 712 mg.

The highest concentrations ranged from 0.6 μg/mL to 24.8 μg/mL and the mean (5.4 μg/mL) was the same as in the intravenous study (Table 2). However, the cumulative dose before the concentration reached 1.0 μg/mL ranged from 146 mg to 1106 mg (mean 551 mg), which was substantially more than in the intravenous study. An example of the time course in one patient illustrating this delay is shown in Figure 6.

Inadequate samples were available for analysis of terminal phase pharmacokinetics. No correlation could be demonstrated between alemtuzumab concentrations and clinical responses because nearly all of the patients (18/20) were responders (CR or PR), and levels of MRD were not reported.

Antibodies following intravenous treatment

A total of 519 serum samples from 30 patients treated with intravenous alemtuzumab were tested for antibodies. These included all samples taken more than 24 hours after the last dose and representative pre-dose samples during treatment. All of them were below the limit of detection (488 U/mL). In 12 patients there were no or very few samples available after the end of treatment, and in the other 18 patients samples were only available between 2 and 6 weeks after the last dose. Therefore, we cannot completely exclude the possibility of delayed responses.

Antibodies following subcutaneous treatment

A total of 281 samples were tested from 21 patients (one patient had adequate late samples for antibody measurement, but none suitable for pharmacokinetic analysis). These included all samples taken after the last dose and representative samples taken during treatment. There were 4 patients who had no or few samples after the end of treatment, but 17 who had adequate posttreatment samples. All samples except those described below were below the LOD.

One patient (no. 8027) had a single sample taken at day 238 (114 days after the last dose) that gave a weak signal just above the LOD. All previous and subsequent samples were less than the LOD. The patient made a good clinical response to therapy, achieving a CR, and no relevant adverse effects were noted. It is possible that the patient made a very weak, transient response to the alemtuzumab, but we doubt that it could have been clinically significant.

One patient (no. 8036) showed a very high titer response, reaching a peak of approximately 3.7 × 10^6 U/mL 24 days after the last dose of alemtuzumab (Figure 7). This anti-IgG1 bound strongly to CAMPATH-1G, the parental rat antibody from which alemtuzumab was humanized, but did not bind other human IgG1.
monoclonal antibodies. Therefore, essentially all of the response was directed against the alemtuzumab idotype. No free alemtuzumab could be detected throughout treatment and there was no significant depletion of tumor cells during therapy. This patient developed a pronounced injection site reaction including edema, erythema, and slight local pain but otherwise no marked general symptoms. As a result, the patient did not achieve the 30-mg dose level during 6 weeks of subcutaneous dosing and alemtuzumab was subsequently administered intravenously. Due to nonresponsiveness, the patient was subsequently treated with fludarabine and went into a long-lasting partial remission. He was restaged 18 months after the end of alemtuzumab treatment (continuing in PR) and still had a high-tier antiglobulin (approx. 1.8 × 10^5 U/mL). There was a very low anti-alemtuzumab signal in one of two samples taken before therapy. This patient might have had a natural anti-IgG (rheumatoid factor) prior to therapy and perhaps was predisposed to make an antiglobulin response.

A search was made for samples from other patients who had received subcutaneous alemtuzumab. Samples were collected between 4 to 50 months after the end of treatment from an additional 9 patients of the original subcutaneous study plus 2 others who received subcutaneous alemtuzumab as part of a compassionate program. A substantial response was detected in patient no. 8020 of approximately 4800 U/mL at 32 months after the end of treatment. Like the response in patient no. 8036, this response cross-reacted on the parental rat antibody but not on other human antibodies, showing it to be essentially anti-idotype. The patient had not responded to the therapy, but was reported to have prolonged skin reactions to the infusions. A very weak response was detected in one other patient of 541 U/mL, barely above the LOD. Unlike the other 2 patients, this serum cross-reacted with other human IgG but not with CAMPATH-1G. We consider that it is a nonspecific rheumatoid-like factor and probably had no clinical significance with respect to alemtuzumab.

In total, therefore, of 32 patients treated with subcutaneous alemtuzumab (30 who were previously untreated with any anti-CLL agent), 2 produced a significant antiglobulin response, which persisted at potentially neutralizing concentrations for more than a year. Both of these patients had received alemtuzumab manufactured at the Therapeutic Antibody Centre, as did most of the patients in this study, although patient no. 8036 had been switched to the commercial product after 3 weeks.

**Discussion**

Alemtuzumab is extensively used for patients with B-CLL who have failed treatment with alkylating agents and purine analogues and investigators are exploring its use in other disease settings. To enable safe and effective use it is essential to understand alemtuzumab's pharmacokinetics and biodistribution as well as the potential for anti-idotype responses, which all may differ according to disease, patient status, and route of administration. We have started to address these issues by comparing blood concentrations of

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*Some doses for patients no. 8033 and no. 8036 were given intravenously due to infusion site reactions during the subcutaneous administration.
alumtuzumab and antilglobulin responses in 2 different studies for treatment of CLL, namely intravenous treatment of chemotherapy-resistant disease and first-line subcutaneous treatment. The studies were initiated independently and had different objectives; nevertheless the pharmacologic results are instructive.

Alumtuzumab may cause substantial "first dose" reactions as a result of cytokine release when it is given intravenously. 3, 14, 19 Therefore, the standard treatment regimen involves dose escalation and frequent, relatively small doses, though it has not been formally established whether the reactions are strictly dose related. A treatment course for chemotherapy-resistant CLL consists of approximately 1 gram of the antibody. However, a cumulative dose of 40 mg to 100 mg is highly immunosuppressive and clinically effective in patients without bulky tumors, for example those treated for prevention of transplant rejection, 15, 20 multiple sclerosis, 21 and other autoimmune diseases. 22-24 as well as to eradicate detectable minimal residual disease in CLL. 23 The biodistribution of alumtuzumab is likely to depend on the bulk of tumor cells, possibly more so than other monoclonals because CD52 is such a highly expressed antigen. There are approximately 5 x 10^9 binding sites for alumtuzumab on a normal lymphocyte. 26, 27 There are about 10^12 lymphocytes in a healthy adult. 28 At least 125 mg of alumtuzumab is therefore required to saturate all of the CD52 sites. CLL cells express a similar amount of CD52 antigen to normal lymphocytes 29 but their total number may be 10 times greater. Therefore, more than 1 g of alumtuzumab would be required to saturate all the receptors in some patients, especially considering that the antibody has a relatively low affinity and that there will be a significant clearance during a 12-week course of treatment. Hence, it is not surprising that the biodistribution and clearance of antibody during the treatment of patients with CLL is dominated by the tumor burden and cannot be fitted to a simple pharmacokinetic model.

There was a strong correlation between the concentrations of antibody throughout intravenous treatment and the ultimate clinical response, or the achievement of minimal residual disease (MRD) negativity in the bone marrow by flow cytometry. It has recently been reported that survival correlates better with MRD than with the standard NCI criteria for clinical responses. 30 Without a good measure for the initial total tumor burden it is difficult to distinguish cause and effect. One possibility is that patients with high tumor burden were treated with inadequate amounts of antibody to achieve sufficient receptor site saturation and see a clinical effect. In any event, it appears that measurements of alumtuzumab concentrations after 2 to 4 weeks of treatment might provide an indicator of the ultimate outcome and guide the physician whether to continue, stop, or adjust the dose of antibody. Most of the antibody was cleared within a few days of the final dose in the nonresponders. However, the half-life at the end of treatment in the responders was comparable with those previously reported for patients without bulky tumors.

Accumulation of alumtuzumab in the blood was significantly slower in the subcutaneous study and it took on average about 6 weeks longer to reach 1.0 μg/mL (an arbitrary threshold known to be potentially lympholytic). This could be a slight overestimate because samples were not always collected every week. Nevertheless, the eventual maximum trough concentrations were very similar in both studies. We cannot tell whether the slower accumulation of antibody in the blood was due to less favorable biodistribution, more effective binding to tumor cells, or the slower dose escalation used in the subcutaneous study compared with that used with intravenously treated patients. It should be possible to resolve this by studies of alumtuzumab given subcutaneously to patients not suffering from malignancies (ie, without excess CD52 cells).

Because clinical responses were much more frequent (19% CR and 68% PR), 8 we could not detect a significant correlation between blood concentrations and clinical responses in the subcutaneous study. With only 2 nonresponders in the group of patients we analyzed, the sample was too small to discern any relationship.

Clear-cut results were obtained from the measurements of antilglobulin responses (antihuman antibody [HAHA]). There were no detectable responses at all in the intravenous study (heavily pretreated patients) and only one patient in the subcutaneous study (previously untreated patients) made a strong response, reaching a peak of approximately 3.7 x 10^4 U/mL 3 weeks after the final dose. This was equivalent to 3.7 mg/mL of the monoclonal anti-idiotype standard and represented a substantial proportion of the total lg in the patient. Similar titers were previously reported during treatment of rheumatoid arthritis patients with subcutaneous alumtuzumab 31 when all of the patients (10/10) made a positive response. After screening 11 other patients we found one with a significant antilglobulin titer of 4800 U/mL. Considering that this sample was taken 32 months after the last treatment with alumtuzumab, it seems likely that this patient also had made a very strong response.

Previous studies on antilglobulin responses to alumtuzumab have given widely different results. In 12 patients treated for kidney transplant rejection no antilglobulins were detected, in contrast to 15 of 17 patients treated with the parental rat antibody CAMPATH-1G. 31 In 167 patients treated for chemotherapy-resistant lymphoid malignancies there were only 3 weak or borderline responses. However, out of 115 patients treated for rheumatoid arthritis, there were 59 positive responses (ILEX Pharmaceuticals, unpublished data on file, July 2003), some of high titer similar to that reported here for patient no. 8036. 19 Many factors could affect the immunogenicity of alumtuzumab, including the dose and length of treatment, the underlying disease, prior exposure to chemotherapy, concomitant immunosuppressive drugs, and possibly the route of administration or the formulation of the drug. Subcutaneous administration might allow more effective antigens presentation, for example, by Langhans cells and with less "excess" antibody circulating to provide tolerogenic signals, the balance might be tipped toward an immune response. From our present study we cannot say whether subcutaneous administration in CLL is more immunogenic than intravenous administration since there were other differences between the 2 groups, most notably the subcutaneous group had received no prior chemotherapy. Even if the 2 groups had been comparable, the difference in responses (0/30 vs 2/32) did not reach statistical significance. This issue needs to be investigated in larger numbers of patients. Both patients who made an antilglobulin response had been treated with alumtuzumab manufactured at the Therapeutic Antibody Centre. We are aware that the manufacture, formulation, or storage of biologics might affect their immunogenicity, but so far we have no evidence to suggest that there is a major difference between the academic or commercial source since antilglobulin responses have been reported in other patients treated with alumtuzumab from either source. The 2 patients (no. 8020 and no. 8036) who produced anti-alumtuzumab antibody on subcutaneous treatment, unlike the other patients, did not show significant reductions in lymphocyte counts but had marked local skin reactions which did not diminish with continued therapy. An antilglobulin response should be considered when there is no drop in the blood lymphocyte count and/or there are persistent injection site reactions.
Alentuzumab provides a useful therapeutic option for patients with CLL. It has excellent activity as first-line treatment and can give good responses in patients who are unresponsive to conventional chemotherapy. The optimal dose route and regimen are not yet known. However, the subcutaneous route can deliver concentrations of antibody comparable to the original intravenous route. The dominant factor influencing biodistribution and pharmacokinetics appears to be the tumor burden. With intravenous dosing, serum concentrations of alentuzumab can provide an early prediction of the ultimate response and might be useful to guide dose adjustment to improve responses or reduce side effects. Strong anti-idiotypic responses may occur in a small minority of patients. Although not associated with serious adverse effects, such responses neutralize the therapeutic benefit and therefore patients with a known anti-idiotypic response should not be further treated with this monoclonal antibody.

Acknowledgments

We are very grateful to the staff of the Therapeutic Antibody Centre, Oxford, United Kingdom, for the manufacture and supply of some of the alentuzumab used in this study. We thank Peter Bone, Ian Clements, and the team at ILEX Oncology for constructive comments and for sharing unpublished data.

References

THE CAPE TOWN EXPERIENCE WITH HAEMATOPOIETIC STEM CELL TRANSPLANTATION IN CHILDREN II: A PRIVATE SECTOR PROGRAMME

Peter Jacobs, Lucille Wood, Jo Lund, June Juritz. The Bone Marrow Transplant Unit – Department of Haematology, Constantiaberg Medi-Clinic; The Division of Clinical Haematology – Department of Internal Medicine, Stellenbosch University-Tygerberg Academic Hospital, Cape Town; Department of Mathematical Statistics, University of Cape Town.

Introduction: Following 5 years of development in a rabbit model traditional bone marrow transplantation in adults was started in 1978 and extended to children in 1986. Our initiative at Groote Schuur Hospital has recently been updated reporting outcome in 28 individuals under 18 years of age. A separate program, outside the state hospital, was activated in 1995. Here the prototype ex vivo T-cell depletion, using Campath monoclonal antibodies, was retained with indications extended to treating immunodeficiency, liposomal storage disease and the haemoglobinopathies. Increasingly donor source includes matched unrelated volunteers. Concurrently oral busulphan has been replaced by the intravenous formulation in preparative regimens. Materials and Methods: With appropriate informed consent consecutive individuals, having a median age of 11 (range 1 – 18), were entered on approved protocols. Outcome was reported and audited by the International Bone Marrow Transplant Registry. Mobilised peripheral blood mononuclear cells were harvested using standardised apheresis technology. Autologous (group I: n=20), allogeneic siblings (group II: n=38) or matched unrelated allografts (group III: n=10) were characterised for mononuclear and CD34+ populations in addition by clonogenic assay prior to infusion with recipients conditioned using a uniform regimen of busulphan and cyclophosphamide. Radiotherapy was reserved for those with high bulk lymphoid tumours. Cyclosporin A was given only to group III with titrated dose reduction over 1 year. Stabilised human serum and viral prophylaxis were discontinued after 3 months whereas that for pneumocystis with cotrimoxazole continued for 1 year. Outcome for age, disease category and transplantation technique was subject to Kaplan-Meier analysis in 68 procedures carried out in 64 patients. Results: Current survival for myeloid leukaemia (n=11) is 73%, the lymphoblastic variant (n=14) is 43%, chronic myeloproliferative syndrome (n=7) is 86%, lymphoma (n=5) is 80%, thalassaemia and haemoglobinopathies (n=6) is 100%, acquired bone marrow aplasia (n=6) is 60%, Fanconi anaemia (n=11) is 55% and miscellaneous group including solid tumours (n=6) is 83%. Additional individuals with immunodeficiency and liposomal storage disease are alive and well. Non-engraftment occurred in 1 of group III and he was successfully retransplanted. Late presenting acute graft-versus-host disease occurred in 3 and responded to topical steroids. Transplant related mortality (n=10) was 15%. Four patients (6%) had cytomegalovirus isolated and pre-emptive ganciclovir prevented progression to disease. Survival was shown to be influenced only by age and disease category. Conclusion: Children can clearly be managed both safely and cost-effectively as part of a well-structured adult programme. Conditioning with intravenous busulphan is now standard having replaced the oral equivalent because of greater recipient acceptability and more reliable pharmacokinetics. Post-transplant mucositis has been virtually abolished by the use of oral sulphate. Currently cyclosporin A is being evaluated in matched unrelated allografts to establish whether it significantly influences any aspect of transplant outcome.

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Professor Peter Jacob,

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for 19th annual meeting of the EBMT: Germsich-Partenkirchen, Jan 17-21, 1993.

Control of GVHD and graft rejection with the monoclonal antibody CAMPATH-1G

Geoff Hale and Herman Waldmann for the CAMPATH users group

T cell depletion of donor bone marrow is the most effective means of preventing GVHD. Unfortunately, it creates an increased risk of graft rejection and, in some cases, of leukaemia relapse. Within the collaborative group of CAMPATH users, we have studied the possible role of CAMPATH-1G (rat IgG2b) for both eliminating the donor T cells and helping to deplete residual T cells from the host. The original method of T cell depletion used CAMPATH-1M (IgM) and human complement to lyse the T cells. In 490 patients transplanted for leukaemia from matched siblings, the incidence of graft failure (or rejection) was 17%. Various studies to reduce graft failure (eg use of post-transplant CyA, total lymphoid irradiation) resulted in only small improvements. Interim results of an ongoing study using CAMPATH-1G to treat the recipient are encouraging. A low incidence of graft failure and GVHD was observed and the disease-free survival of the patients appears to be better than before. In other studies, CAMPATH-1G was used to opsonize the donor bone marrow before infusion. In this case excess antibody may contribute to immunosuppression of the host. So far 20 patients have been treated. Only one suffered severe GVHD but two suffered graft rejection, suggesting there is still some residual host immunity. Current studies focus on the use of CAMPATH-1G both in vivo and ex vivo to control both problems.
CAMPATH-1 Monoclonal Antibodies in Bone Marrow Transplantation

GEOFF HALE and HERMAN WALDMANN for CAMPATH USERS

ABSTRACT

CAMPATH-1 (CDw52) antibodies recognize a very small lipid-anchored glycoprotein that is expressed on the surface of human lymphocytes. They are remarkably lytic with human complement. In addition, CAMPATH-1G (rat IgG2a) and CAMPATH-1H (human IgG1) bind to human Fe receptors and are very effective for cell lysis in vivo. CAMPATH-1M (rat IgM) and CAMPATH-1G have been used to control GVHD and graft rejection in bone marrow transplantation by depletion of the T cells of the donor and recipient. Depletion of donor T cells alone gave excellent control of GVHD but up to 20% of the patients transplanted from HLA-matched siblings, and 51% of those transplanted from nonsibling donors, experienced graft failure caused by immunological rejection. Graft rejection could be partly overcome by additional immunosuppression either with CsA or total lymphoid irradiation (TLI). More effective was the use of CAMPATH-1G in vivo to deplete residual host lymphocytes. Preliminary results from current protocols of antibody depletion give two year actuarial leukemia-free survival as good as or better than similar studies with conventional GVHD prophylaxis, as well as a decreased morbidity from chronic GVHD, although engraftment was delayed by about 5 days. We propose that prophylactic T cell depletion with CAMPATH-1 antibodies is a simple and valid alternative to drug-based immunosuppression that may be particularly applicable to patients with acute leukemia or nonmalignant diseases transplanted from HLA-matched siblings as well as any patients transplanted from unrelated donors. Future developments of antibody-based immunosuppression may allow the extension of marrow transplantation for tolerance induction to organ transplants or in autoimmune diseases.

INTRODUCTION

Bone marrow transplantation can be used in the management of several different kinds of disease. It gives a remarkably powerful effect against some types of leukemia (notably CML) and would often be the treatment of choice for inborn or acquired defects of the hemopoietic system. In theory, it could be used to create tolerance to other organs and as a radical treatment for severe autoimmune diseases. The biggest problem is the two-way immune recognition of donor and recipient that leads to the major complications of graft-versus-host disease (GVHD) or graft rejection. With powerful immunosuppressive drugs, these can be controlled to some extent, yet GVHD still remains a significant cause of morbidity even when the donor and recipient are HLA-matched siblings. When the donor and recipient are less well matched, or unrelated, GVHD and graft rejection are major problems and this currently limits the use of marrow transplantation to only the most severe diseases. The key players in the generation of immune responses are T lymphocytes, and it has been shown that they are the prime mediators of...
GVHD and rejection. If T cell alloreactivity could be more effectively controlled, then the immunological complications could be overcome and marrow transplantation could be more widely applied.

Several years ago it was shown in animal models that removal of mature T cells from the donor bone marrow would prevent GVHD (1–4). However, ablation of recipient T cell function has not been so straightforward. Even after total body irradiation some recipient T cells remain and, in the absence of donor T cells, they can gain the upper hand and reject the marrow (5–7). The methods that were originally developed for depleting the donor T cells (e.g., agglutination with soybean lectin, E-rosette, density gradient centrifugation) were mostly not applicable to depletion of the same cells in vivo, so it was difficult to tackle this side of the problem with any specificity. The one type of agent that could be used both in vitro and in vivo was antilymphocyte globulin, and this was shown to be immunosuppressive and capable of preventing GVHD (8). The problem with antilymphocyte globulin is that it is a heterogeneous mixture of antibodies directed against different cell antigens, only a minority of which are T cell specific. It was therefore difficult to obtain samples that were reproducibly active and nontoxic.

When monoclonal antibodies became available, one of the first clinical applications was for purging marrow of donor T cells. However, most monoclonal antibodies were singly very inefficient at killing target cells because they did not activate human effector mechanisms (complement and/or Fc-receptor-dependent cell-mediated killing). Early efforts were therefore not very successful (9,10) and this led many investigators to search for different ways of using the antibodies for cell depletion. One method was to couple the antibody to a toxin (e.g., ricin) (11); others have used magnetic particles (12) or immunoadsorption (13). However, we continued to look for antibodies that could exploit natural human effector functions, with the prospect that these could eventually be used in vivo as well. Thus finding a lytic antibody for T cell depletion of bone marrow was not just an end in itself, but also a way to test reagents that might later find wider applications for immunosuppression.

THE CAMPATH-1 ANTIGEN

From a fusion of rat spleen cells with a rat myeloma cell line, we selected a set of antibodies that was unusually lytic for human lymphocytes using autologous complement (14–16). It turned out that all of them recognized the same antigen, which we now know to be a glycoprotein that is attached to the cell membrane by a lipid (glycosylphosphatidylinositol, GPI) anchor (17,18). Most of the antibodies were IgM or IgG2a, but just one was a rat IgG2b. This contrasted with antibodies against other cell surface antigens isolated from the same fusion, which were almost exclusively IgG2a or IgG2b. The CAMPATH-1 antibodies recognized virtually all human lymphocytes, both T cells and B cells as well as monocytes and macrophages, although the latter seemed to be less sensitive to complement-mediated lysis. Tests on panels of fresh leukemia and lymphoma cells and cell lines showed that they recognized most cases of lymphoid malignancy, but only a small minority of myeloid leukemias (17,19). In the fourth leukocyte workshop the CAMPATH-1 antibodies were assigned to the cluster CDw52 (20). Little or no reactivity with colony-forming cells could be detected either by complement-mediated lysis, or by cell sorting (14,15). This important result was confirmed in numerous laboratories (21–24), although one group has reported only approx. 40% recovery of colony-forming cells (25). Valentin and co-workers (26), using a different CDw52 antibody, which is known to have a broader reactivity than CAMPATH-1M, also found a significant depletion of CFU-GM. In these cases, we cannot be sure that the colony-forming cells did not require growth factors from the lymphocytes since the appropriate controls were not included. However, a recent comprehensive study of CAMPATH-1H found no evidence for reactivity with the cells that give rise to colony-forming cells during long-term culture (27).

CAMPATH-1 antibodies were found to recognize lymphocytes from old world monkeys, but not other species (15,23). At this time we also found that red cells were recognized in some of the monkeys. Antigen expression on the red cells seems to be under the control of a single autosomal gene, whereas expression on lymphocytes is constitutive (28). It has been reported that some CAMPATH-1 antibodies can recognize an antigen on human red cells (26), but despite extensive studies with large panels of cells using numerous techniques, we have never been able to detect such reactivity (15) (V. Taylor and G. Hale, unpublished observations).

Structure of the antigen

Recent work indicates that the antigen is a remarkably small glycoprotein, having only 12 amino acids, with a single N-linked carbohydrate on Asn-3, which is comparatively large and complex (Fig. 1) (18,29,30). At the C-terminus the peptide is attached to a glycosylphosphatidylinositol anchor that has a conventional structure similar to other mammalian GPI anchors. The N-linked carbohydrate, and indeed the 9 N-terminal amino acid residues, can be removed without destroying the antigenicity or sensitivity to complement lysis. The CAMPATH-1 antibodies therefore seem to recognize an epitope which includes the three or four C-terminal amino acids and possibly part of the GPI anchor. This implies that they bind very close to the cell membrane, which
could be one of the reasons why the antibodies are so good for cell lysis, since one of the critical events in the complement cascade is the covalent attachment of activated C4 and C3 to the cell membrane. These molecules have a very short half-life when activated and so could easily decay before reaching the membrane if activation by antibody bound C1 occurred at a greater distance from the lipid bilayer.

However, there is another plausible explanation why GPI-anchored antigens, in general, could be good targets for complement attack, since we now believe that they are organized into clusters on the cell surface (31,32). The first component of complement, C1, needs to bind to at least two antibody molecules to become activated and so the density and distribution of the antigen is critical (33–35). Even abundant antigens can be poor targets for lysis, but there is a dramatic improvement when two monoclonal antibodies are used that recognize different epitopes (36,37). This results in a high frequency of antibody pairs suitable for binding C1. It is possible that the same thing occurs with single monoclonal antibodies against antigens that naturally occur in clusters.

There are several other examples of antigens that are good targets for complement lysis and that have proved to be good targets for therapy (38–44) [reviewed by Xia (42)]. Most of them are glycolipids or small GPI-anchored glycoproteins, in which case either of the above explanations could be relevant. The importance of these observations for bone marrow purging is that not all target antigens will be appropriate for efficient complement-mediated attack, irrespective of the isotype of the antibody.

Expression of antigen on spermatozoa

There is very little information about the physiological function of the CAMPATH-1 antigen. Like virtually all other GPI-anchored glycoproteins, there is some evidence that cells can be activated by CAMPATH-1 antibodies under suitable conditions (17,26,43). However, an intriguing observation was the discovery of the same antigen on mature human sperm (44–47). It actually seems to be synthesized by the epithelial cells of the epididymis, secreted into the seminal plasma (probably in the form of membrane-bound vesicles) from where it is taken up by the sperm as they pass through. Mature sperm are therefore sensitive to complement attack in the presence of CAMPATH-1 antibodies, but this is not thought to be a major problem in the context of therapy because only minute amounts of antibody would be expected to reach the seminal fluid and there the sperm would be protected by the large excess of vesicular antigen (47).

A homologous epididymal antigen has recently been identified in monkeys (48). It has a mature protein sequence of only 11 amino acids, which is very similar to the human antigen. It is likely that this is the same antigen that we detected on monkey lymphocytes. In mice, a related antigen has also been described that is expressed on lymphocytes and epididymis (49,50). Although the mature peptide (approx. 20 amino acids) does not closely match the human antigen, it contains a similar N-glycosylation site and the untranslated portions of the cDNA are significantly similar. We expect that these homologues will provide good targets for experiments to elucidate the antigen function. Meanwhile, they will also provide models for studying the therapeutic applications of CAMPATH-1 antibodies, which are by now well advanced in humans.

APPLICATIONS OF CAMPATH-1 ANTIBODIES IN BONE MARROW TRANSPLANTATION

CAMPATH-1M for T cell depletion

CAMPATH-1M was selected because it gave the highest titre of cell lysis with no effect on colony-forming cells. Studies in cynomologous monkeys and in patients with end-stage lymphocytic leukemia showed no unexpected toxicity (51) and experiments in monkeys also showed no delay in the engraftment of autologous bone marrow treated with CAMPATH-1M and complement in vitro (52). On the basis of these studies, colleagues at the Hammersmith Hospital, London, the Hadassah University Hospital, Jerusalem, and the University Hospital, Ulm, started to use CAMPATH-1M with complement from donor serum for T cell depletion of donor bone
marrow for allogeneic transplants. A series of 11 patients transplanted for advanced leukemia from HLA-matched siblings demonstrated that this was a very effective method of preventing GVHD, even when no posttransplant immunosuppression was used (21). Nevertheless, even in this small series, three cases of graft failure were observed, which we now know is the almost inevitable corollary of extensive T cell depletion.

Several lines of evidence suggest that the graft failure was mainly due to immunological rejection of the donor bone marrow, rather than, for example, an adverse effect of the antibody treatment on stem cells or their ability to repopulate the recipient (53). First, only one case of graft failure has been seen in more than 60 patients who received autologous bone marrow purged by the same method (to remove residual leukemia cells). Second, the incidence of graft failure was higher when bone marrow from unrelated or mismatched donors was used. Third, there were several cases in which host-derived cytotoxic T cells could be detected immediately preceding graft failure and some of them were shown to be specific for donor cells (54–56).

Since 1983, more than 500 transplants have been performed by 23 transplant centers throughout Europe and the Middle East, using this method of preventing GVHD in HLA-matched siblings (22,57–63) (Protocol 01, Fig. 2). Various modifications to the regime were made in an effort to reduce the risk of graft rejection. Some patients received posttransplant CsA. Others received intensified conditioning regimes, including additional radiotherapy, drugs, or total lymphoid irradiation.

A recent analysis (64) in patients transplanted for malignant diseases showed that the incidence of either severe or chronic GVHD was substantially reduced compared with other types of therapy. Overall, the frequency of acute GVHD > grade 1 in patients at risk was 17% and the frequency of severe acute GVHD (grade 3–4) was only 7%. The frequency of chronic GVHD was 19% and of severe chronic GVHD, 2%. The incidence of graft failure was lower in those patients who received CsA or TLJ (12–13%) compared with those who did not (19–21% graft failure). The use of CsA (though not TLJ) was also associated with a significant reduction in transplant-related mortality. Only a small number of patients (n = 24) received both TLJ and CsA, but they fared particularly well with only 4% graft failure.

**Prevention of rejection with CAMPATH-1G**

The partial success in prevention of graft failure by immunosuppressive reagents led us to believe that better control might be achieved with more selective and potent immunosuppressants. Experiments in animals showed that treatment of the recipient with depleting anti-T cell antibodies could abolish rejection of T cell-depleted bone marrow, even with reduced irradiation or when the donor and recipient differed across major histocompatibility barriers (7,65). We therefore looked for monoclonal antibodies that could produce a similar effect in humans. Antibodies that could deplete lymphocytes in vivo were important in these experimental studies and so again we turned to the CAMPATH-1 antibodies since they were the most effective reagents available at the time (66).

The original IgM antibody CAMPATH-1M, although extremely lytic with complement, gave only transient depletion of lymphocytes in vivo in patients with CLL or lymphoma (51). However, from another CDw52 clone that secreted an IgG2a antibody, we were able to isolate a spontaneous class-switch variant, the rat IgG2a antibody CAMPATH-1G (62). The IgG2a subclass is not only the best rat IgG for activating complement but is also optimal for binding to human Fc receptors and activating cell-mediated killing (67–69). CAMPATH-1G proved to be very efficient at depleting lymphocytes in vivo in several patients with lymphoid malignancies and in patients

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**FIG. 2.** Illustration of the different antibody protocols studied by the CAMPATH users group. The dose of CAMPATH-1M was typically 25 mg for in vitro purging, whereas the dose of CAMPATH-1G was usually 5–10 mg for in vitro purging or 5–10 days at 5–10 mg/day for in vivo treatment.
suffering steroid-resistant kidney graft rejection (70,71). In both studies, the treatment with CAMPATH-1G gave a beneficial clinical response in many of the patients.

The first objective was to see whether treatment with CAMPATH-1G would decrease the risk of graft rejection in the context of "standard" T cell depletion. This was not easy to test because the actual incidence was comparatively low, and so quite a large number of patients was needed to demonstrate an effect. Initially we did not know whether the recipient's effector mechanisms would be compromised by the conditioning regimen, so it was possible that administration of antibody at the time of the transplant might not be effective. Therefore the CAMPATH-1G was given at 5–10 mg/day for 5 days before the start of chemoradiotherapy (Protocol 02). Although this had the slight disadvantage that extra inpatient time was required, the protocol had several advantages: (a) the degree of lymphocytopenia produced by CAMPATH-1G could be measured, uncomplicated by other treatments, (b) adverse effects of the antibody could likewise be assessed, and (c) most of the antibody would be cleared by the time of the transplant and so be unlikely to contribute to extra depletion of the donor T cells. T cell depletion with CAMPATH-1M and other elements of the conditioning regime were kept the same as in historical control groups. No other prophylactic agents to prevent GVHD or graft rejection (e.g., CsA, TLI) were given. The plan is to enroll 100 patients with acute leukemia in first remission, which will provide a homogeneous group where the effect of the antibody protocol on relapse can also be observed.

To date about 75 such patients have been enrolled by three transplant teams (Royal Free, London; University Hospital, Ulm; King Faisal Hospital, Riyadh). The interim results are very satisfactory and a detailed report will be published when the study is complete. Meanwhile we have analyzed these study patients as part of a larger group of patients, including CML and advanced disease, who all received the same antibody protocol (Table 1). Out of 144 patients there has been only 2% severe acute GVHD, no case of severe chronic GVHD, and 9% graft failure. The risk of death at 2 years from all causes other than relapse is 21 ± 8%, which is significantly lower than that seen with the original protocol (38 ± 3%). Furthermore, the leukemia-free survival at 2 years is also significantly better (Fig. 3A). Although graft failure has not been totally eliminated, there is a possibility that in the future, the addition of a short course of CsA might make the risk still lower.

The degree of lymphocyte clearance induced by CAMPATH-1G in vivo has been measured by a sensitive limiting dilution analysis and found to be similar to that achieved with busulphan/cyclophosphamide or total body irradiation (72). Antibody therapy was as effective as the conventional treatments at depletion of cytotoxic T cell precursors (> 99%) but far more effective at eliminating helper T cells that were relatively resistant to chemotherapy or radiotherapy.

The use of CAMPATH-1G for prevention of GVHD

The successful use of CAMPATH-1G to eliminate cells in vivo suggested that it could be used in a similar way to remove the donor T cells in order to prevent GVHD. In principle, this could be done either by addition of the antibody to the donor bone marrow to opsonize the cells for subsequent clearance in vivo (Protocol 03), or by administration to the patient at or around the time of the transplant (protocols 04–06). In both cases, the antibody should also help to remove residual host lymphocytes though its effect on the donor T cells would presumably be greater when it was administered with the bone marrow. The advantage of these procedures is that no additional complement or processing of the bone marrow would be required. Both approaches have been tried by different transplant centers and the results prove that sufficient depletion of donor T cells to avoid GVHD can be achieved by either method (Table 1).

Teams at the Hadassah Hospital, Jerusalem and the University Hospital, Cape Town have used CAMPATH-1G in vivo (Protocol 03) (73–75). Most patients (24/26) received additional total lymphoid irradiation to prevent graft rejection, but no posttransplant immunosuppression. The incidence of either severe GVHD or graft failure was very low, but as the number of patients so far treated is small, this is not yet significantly different from the results with the original protocol. However, the 2 year transplant-related mortality of this group of patients is already significantly better than those treated on the original protocol and so is the leukemia-free survival (Fig. 3B).

These results provide convincing evidence that more opsonization with CAMPATH-1G is sufficient to deplete the donor T cells. We know that CAMPATH-1G can bind to human Fc receptors as well as activating human complement, but we cannot say which mechanism is more important. If the donor bone marrow was not washed free of plasma, significant lysis of T cells was obtained within 30 min of addition of CAMPATH-1G, even though no extra complement was added (W. Fiehe and R. Willemze, University Hospital, Leiden and E. Yousaf and D. Pamphilon, Blood Transfusion Service, Bristol, unpublished observations). However, the procedure seemed to be equally as successful with bone marrow separated on Ficoll and washed free from plasma, so presumably the necessary effector mechanisms are sufficiently intact in the recipient despite the chemoradiotherapy.

An alternative way of using CAMPATH-1G to prevent GVHD has been tested by teams at the University Hospi-
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<td>19</td>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>02</td>
<td>CP1G</td>
<td>CP1M</td>
<td>144</td>
<td>0.019</td>
<td>8</td>
<td>0.032</td>
<td>20</td>
<td>0</td>
<td>0.00018</td>
</tr>
<tr>
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<td>5</td>
<td>10</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>04/6</td>
<td>CP1G</td>
<td>None</td>
<td>41</td>
<td>0.025</td>
<td>8</td>
<td>17</td>
<td>14</td>
<td>0.000124</td>
<td>34</td>
</tr>
<tr>
<td>07</td>
<td>CP1G</td>
<td>CP1G</td>
<td>28</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>Too early</td>
</tr>
</tbody>
</table>

*The fraction who suffered graft failure or GVHD is calculated as a percentage of the patients at risk. Patients were considered not evaluable for engraftment if they died before day 20 without engraftment, and not evaluable for GVHD if they suffered graft failure or died before day 100 without GVHD (day 120 for chronic GVHD). Results in each protocol were compared with the original protocol (01) by the χ² test. Those that are significantly better are in boldface and those that are worse are in italics. Note: Leukemia-free survival means freedom from hematologic disease. Cytogenetic relapse in CML was not included.
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PATH-1M \textit{in vitro}. However, an observation that may be relevant is that all of the patients who have received CAMPATH-1G \textit{in vivo} had slower engraftment than normal, with a median time to reach 500 neutrophils of about 24 days compared to a median of about 19 days for patients who had only \textit{in vitro} T cell depletion (64). This delay in engraftment was more marked when the CAMPATH-1G was given close to or around the time of the transplant. We do not think there was a direct toxic effect on bone marrow stem cells because no delay was seen with CAMPATH-1G \textit{in vitro}. Possibly, a small number of lymphocytes, either donor or host, play a role in engraftment, perhaps by secreting cytokines that promote the growth of progenitor cells.

**Leukemia relapse**

One of the potential risks of T cell depletion for patients with leukemia is the loss of a beneficial graft-versus-leukaemia effect. In 1979 the Seattle transplant team showed that patients with advanced acute leukaemia who suffered from GVHD were at lower risk of relapse, compared with those who did not develop GVHD (78). Subsequent analysis of large collections of data has confirmed this finding in all the major categories of leukaemia treated by marrow transplantation (79). There has been considerable speculation as to whether the beneficial graft-versus-leukaemia effect can be separated from GVHD, perhaps by selective manipulation of the donor T cells, and there have been several experimental studies in animals which suggest that this may be possible (80–83). However, there are very considerable practical difficulties in applying these principles to human therapy. At present, we can only examine whether the trade-off between decreased transplant-related mortality and increased relapse is favorable or not.

The risk of relapse for all patients with early leukemia who received T cell-depleted transplants is shown in Fig. 4. Results of both \textit{in vitro} and \textit{in vivo} depletion are included, but patients who received any form of T cell addback are excluded. With up to 10 years follow-up, the risk of relapse in ALL and AML shows a plateau at approx. 30%, with most cases occurring in the first year. In CML, the risk of hematologic relapse continues to rise for at least 5 years, reaching an apparent plateau of about 60% by 7 years. It has been clear for some time that T cell depletion results in a significant increase in relapse risk for patients with CML transplanted from matched siblings (59,61,62,84,85), though it was initially surprising to find that the risk of relapse of T-depleted patients is much greater than for conventionally treated patients who do not suffer from GVHD (79). This is perhaps the best evidence yet in humans that the antileukemic effect of the marrow transplant might be distinct from clinical GVHD.
HALE AND WALDMANN

In contrast, the results in acute leukemia do not show such a distinction, since the effect of T cell depletion can be accounted for in terms of the improved control of GVHD. The relapse rates we see are a little greater than reported using conventional therapy with MTX/CsA (86.87) and this results in a slightly lower leukemia-free survival when we consider the CAMPATH-1-treated patients as a whole. However, the overall control of transplant-related complications is so much better with the recent protocols, particularly 02 and 03, that this should compensate for the slightly increased risk of relapse. Currently the leukemia-free survival at 2 years for patients with acute leukemia treated according to protocol 02 or 03 is 56%, which is almost exactly what would be expected using MTX/CsA. However, the improved control of GVHD, particularly chronic GVHD, with antibody therapy is likely to be a significant advantage.

Transplants for nonmalignant diseases

If antibodies could be routinely used to control GVHD and rejection, one of their most useful applications would be in transplants for nonmalignant diseases, such as severe aplastic anemia and eventually thalassemia or sickle cell anemia. These situations are not complicated by the potential graft-versus-leukemia effect of donor T cells. Irradiation is also undesirable and so there would be significant long-term advantages in obtaining a powerful but nontoxic immunosuppressive conditioning regimen.

To date the CAMPATH-1 users group have carried out 116 such transplants from HLA-matched siblings (80 aplastic anemia, 22 thalassemia, 9 Fanconi’s anemia, 5 other inborn errors). A diversity of conditioning regimens and antibody treatment protocols has been tested as in the patients with malignant diseases. However, the numbers of patients treated according to each individual protocol are small. Two main groups of patients can be identified according to the antibody treatment (Table 2). The first group received T cell depletion in vitro, either with CAMPATH-1M (31 patients, protocol 01) or CAMPATH-1G (16 patients, 17 transplants, protocol 03). The second group received CAMPATH-1G in vivo either with

![Graphs showing relapse rates for ALL-CR1, AML-CR1, and CGL-CP1 over time.](Image)

FIG. 4. Risk of relapse for patients with early leukemia transplanted from matched siblings. The results are pooled from all antibody protocols, excluding patients who received T cell addback.

### Table 2. Results in Matched Sibling Transplants for Nonmalignant Disease

<table>
<thead>
<tr>
<th>Protocol</th>
<th>No. of patients</th>
<th>Graft fail</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
<th>Actuarial at 2 years ± SE survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>01/3</td>
<td>In vitro</td>
<td>48</td>
<td>0.015</td>
<td>0.02</td>
<td>0.015</td>
</tr>
<tr>
<td>02/05/67</td>
<td>In vivo</td>
<td>20</td>
<td>0.015</td>
<td>0.02</td>
<td>0.015</td>
</tr>
</tbody>
</table>
T cell depletion in vitro (11 patients, protocols 02, 07) or without (9 patients, protocol 06). Three of the transplants in the second group were retransplants after graft rejection of patients in the first group (75). In every case the control of GVHD has been excellent. There were only two cases of acute GVHD ≥ grade 1 and only one case of chronic GVHD (not severe). In the group who received only in vitro T cell depletion, the incidence of graft failure was 45%, but in the group who received in vivo T cell depletion it was only 5% (p < 0.01, χ² test). The actuarial survival at 1 year for the in vitro group was 74% and for the in vivo group 100% (p < 0.03, log-rank test). Unlike the situation in transplants for leukemia, the use of CAMPATH-1G in vivo was not associated with any delay in engraftment. The median day to reach 500 neutrophils for the in vitro group was day 19 and for the in vivo group was day 14 (excluding patients who did not engraft). Unlike the patients with leukemia, most of these patients did not receive TBI. Possibly their bone marrow microenvironment was therefore more conducive to rapid regeneration.

Transplants from donors other than HLA-identical siblings

Only a minority of patients who might benefit from a marrow transplant have a suitable HLA-identical sibling. Patients who receive transplants from HLA nonidentical family members or from "HLA-matched" unrelated donors are at significantly higher risk of GVHD and other severe transplant complications. Therefore much attention has been given to the use of T cell depletion in these situations. CAMPATH-1 antibodies have been particularly used in transplants from parents for immune deficiencies and other inborn errors (88-92) and in unrelated donor transplants (93-95).

A summary of the results to date is given in Table 3. T cell depletion in vitro gave good control of GVHD with only 25 cases of severe acute GVHD out of 180 patients evaluable (14%). However, the incidence of graft failure was substantial (51%) in those patients who did not receive additional therapy with CAMPATH-1G in vivo. This is despite the fact that many of them received various other treatments designed to reduce the risk of graft rejection, such as additional irradiation or CsA or other antibodies, including LFA-1 and CD2 (92). Patients who received in vitro T cell depletion combined with in vivo treatment with CAMPATH-1G had a significantly lower incidence of graft failure (24%), though it was still a substantial problem. The use of in vivo antibody alone combined with conventional GVHD prophylaxis using MTX/CsA gave a similar rate of graft failure and GVHD.

Because these results included all categories of patients with early or advanced leukemia or nonmalignant disease, it would be uninformative to compare the survival curves. The largest subset is the patients transplanted for CML in chronic phase from unrelated donors (94). They are of particular interest in view of the high incidence of relapse that was seen following T cell depletion in HLA-matched sibling transplants. The patients are divided into two groups (Table 4). Thirty patients received in vivo CAMPATH-1G combined with in vitro T cell depletion (Protocol 02) and 60 received only in vivo CAMPATH-1G with conventional GVHD prophylaxis (Protocol 05/06). There were no significant differences between the incidences of graft failure and GVHD in the two groups although, as expected, the trend was toward less GVHD but more graft failure with in vitro T cell depletion. However, in both cases the incidence of relapse seemed to be much lower than in comparable transplants from HLA-matched siblings (Fig. 5). Two-year survival was significantly better in the group who received only in vivo antibody, and the

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**Table 3. Results in Noneibling Transplants for Malignant and Nonmalignant Diseases**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>No. of patients</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
<th>Either</th>
<th>Actuarial at 2 years ± survival</th>
</tr>
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<tr>
<td></td>
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<tr>
<td>HIV matched family members and unrelated donors</td>
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</tr>
<tr>
<td>01/3 T depleted</td>
<td>27</td>
<td>44</td>
<td>13</td>
<td>31</td>
<td>42</td>
</tr>
<tr>
<td>02 T depleted + CPG in vivo</td>
<td>125</td>
<td>0.02</td>
<td>22</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>05/6 CPG in vivo (pre- and post-Tx)</td>
<td>110</td>
<td>0.02</td>
<td>23</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>HLA mismatched family members</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>01/3 T depleted</td>
<td>124</td>
<td>54</td>
<td>16</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>02 T depleted + CPG in vivo</td>
<td>40</td>
<td>0.02</td>
<td>32</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>05/6 CPG in vivo (pre- and post-Tx)</td>
<td>7</td>
<td>29</td>
<td>20</td>
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<td>25</td>
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23
<table>
<thead>
<tr>
<th>Protocol</th>
<th>No. of patients</th>
<th>Graft fail</th>
<th>Acute GVHD</th>
<th>Chronic GVHD</th>
<th>Either</th>
<th>Transplant mortality</th>
<th>Relapse</th>
<th>Leukemia-free survival</th>
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<td>3/4</td>
<td>M</td>
<td>S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA matched siblings</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01/3 T depleted</td>
<td>150</td>
<td>18</td>
<td>11</td>
<td>7</td>
<td>23</td>
<td>0</td>
<td>32 ± 5</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>02 T depleted + CP1G in vivo</td>
<td>27</td>
<td>11</td>
<td>13</td>
<td>4</td>
<td>55</td>
<td>0</td>
<td>31 ± 27</td>
<td>9 ± 19</td>
</tr>
<tr>
<td>Unrelated donors</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>02 T depleted + CP1G in vivo</td>
<td>30</td>
<td>27</td>
<td>39</td>
<td>9</td>
<td>40</td>
<td>7</td>
<td>67 ± 9</td>
<td>4 ± 7</td>
</tr>
<tr>
<td>05/6 CP1G in vivo (pre- and post-Tx)</td>
<td>60</td>
<td>12</td>
<td>31</td>
<td>19</td>
<td>36</td>
<td>9</td>
<td>0.0040 ± 15</td>
<td>17 ± 14</td>
</tr>
</tbody>
</table>

Table 4: Results in Transplants for CML in First Chronic Phase
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FIG. 5. Relapse after T cell-depleted transplants for CML. Comparison of unrelated and matched sibling donors. The results are pooled from all antibody protocols, excluding patients who received T cell addback.

leukemia-free survival in this group (53 ± 15%) was as good as that obtained in matched sibling transplants and superior to published results for unrelated donor transplants using conventional GVHD prophylaxis (96).

Lymphoproliferative disease, immune reconstitution, and infections posttransplant

Unlike some other trials of T cell depletion (97–100) lymphoproliferative disease was rare. Out of 1529 transplants, 10 cases of lymphoproliferative syndrome or secondary lymphoma have been reported (101). This represents an actuarial risk at 5 years of 1% in transplants from siblings (5/1067) and 2.3% from mismatched or unrelated donors (n = 5/462). CAMPATH-1 antibodies recognize B cells as well as T cells and it is possible that bone marrow purging with CAMPATH-1M or CAMPATH-1G helps to reduce the potential target for EBV-driven oligoclonal proliferation of B cells in the early posttransplant period, which can lead to fatal malignant lymphomas if uncontrolled.

The CAMPATH users group have not centrally collected data on the recovery of lymphocyte subsets or the incidence of nonfatal infections because of the huge amount of complex reporting and analysis this would have entailed. The number of fatal infections late after transplant did not seem to be more than seen with conventional GVHD prophylaxis, since transplant-related mortality did not increase much after the first 9 months. However, individual centers have observed relatively slow T cell regeneration following lymphocyte depletion with CAMPATH-1M (102,103) and some have found a high incidence of CMV viremia (A Bacigalupo, Genoa, and D. Bunjes, Ulm, unpublished observations). Since the normalizations of T cell numbers may not occur for a year or more after a bone marrow transplant even with conventional GVHD prophylaxis, it is not easy to assess the impact of T cell depletion on this parameter. Furthermore, we do not know how well the T cell phenotype really reflects the immune status of an individual. Probably functional assays of T cells are more relevant although they are more laborious to carry out.

CONCLUSION

T cell depletion is certainly the most effective method of preventing graft-versus-host disease. We have developed simple procedures to accomplish this using the patient's own immune effector mechanisms. CAMPATH-1 monoclonal antibodies are particularly effective, possibly because of the unique properties of their target antigen. Furthermore, there is the possibility that in vivo antibody treatment may contribute to elimination of residual leukaemia in some cases (70).

Better control of GVHD is a prerequisite before bone marrow transplantation can be fully used in the treatment of a wide range of diseases. Unfortunately, T cell depletion is associated with increases in the risks of graft rejection and leukemia relapse. Substantial progress has been made toward overcoming the problem of graft rejection by giving increased immunosuppression to the recipient, either with CsA or TLI, or most successfully by in vivo treatment with CAMPATH-1G. Eventually we believe that a combined strategy should permit successful engraftment in virtually all HLA-matched sibling transplants and the great majority of others. This should mean that bone marrow transplants can be used for nonmalignant hematological diseases with less fear of procedure-related mortality and it might open the way to the use of marrow transplantation for tolerance induction in organ transplantation and autoimmune disease.

The risks of leukemia relapse associated with extensive T cell depletion have now been well defined, at least in patients with early disease. In acute leukemia the increase in risk is modest compared with current results using drug-based GVHD prophylaxis. The extra mortality from relapse is likely to be offset by a reduction in transplant-related complications. In CML, T cell depletion has a significant adverse effect, at least in patients transplanted from HLA-matched siblings. We do not know why a similar increase in relapse was not seen in patients transplanted from unrelated donors. It may be relevant that most of the latter group receive higher doses of radiotherapy (95), but it is also possible that there is a stronger allogeneic effect that is not completely abrogated by the antibody depletion.

Some groups have tried to add back small numbers of lymphocytes to the depleted bone marrow or after the transplant in order to preserve a graft-versus-leukemia effect (74,104,105). The effect has been to increase the risk of GVHD and decrease the risk of leukemia relapse.
(64) and, overall, the results are similar to those obtained without T cell depletion. In other cases donor T cell infusions have been used to effectively treat outright relapse (106, 107), but this procedure is not always effective and carries a high risk of fatal GVHD. Ideally, T cell addback could be administered 2–4 months after the transplant when the patient had recovered from the effects of the conditioning regime but before hematological relapse is likely to have occurred. If a sensitive assay were available to indicate the amount of residual disease at that stage (108), this immunotherapy could then be reserved for only those patients who were likely to relapse and had not suffered any GVHD. However, whatever refinements are made in the timing, dose, or cellular content of lymphocyte therapy, it is unlikely that its potential for producing severe GVHD will be eliminated. The biological effect will largely be dictated by the immune repertoire of the lymphocytes and the genetic disparity between donor and recipient. The alternative strategy is to seek for new drug treatments for residual leukemia, or more effective combinations of existing ones. For example, the drug thiota pera is reported to be myelosuppressive and may be useful for treatment of CML in the context of T cell depletion (109, 110). So far the clinical results are very early, and longer follow-up is needed to be sure whether this treatment will give improved results.

Bone marrow transplantation will continue to be an important arena for the testing of new types of biological therapy. As the clinical results gradually improve and the range of potential new treatments become wider, it will be more and more difficult to design prospective trials that have a good chance of showing significant improvements. We believe that the best use can be made of these limited opportunities if we test therapies that, like CAMPATH-1, potentially have a very broad application. Although we have not described here the wider range of clinical applications where CAMPATH-1 antibodies are being used, such as for treatment of leukemia, control of organ transplant rejection, and treatment of autoimmune diseases (70, 71, 111–114), nevertheless, the results obtained from the studies in bone marrow transplantation have helped to make those possible.

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disease and graft rejection after bone marrow transplan-

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number of patients suffered from EBV-B lymphoproliferative disease after receiving an IR-depleted graft (6 out of 12 patients), whereas the control group had limited EBV-SLPOD (3 out of 20 patients). This might be caused by the effect of SBE in the control group, resulting in an additional B cell depletion. So, a new study is currently being performed in which B cells are also depleted, using CD19 and CD22 tetrameric complexes, in order to avoid this complication.

750 IMMUNOMAGNETIC SELECTION OF PERIPHERAL BLOOD STEM CELLS (PBSC) IN HAEMATOLOGICAL MALIGNANCIES


Hospital Universitario de Canarias, Tenerife, Spain

Apheresis harvests from patients with haematological malignancies can be purged with immunomagnetic methods, yielding an important enrichment in CD34+ cells and simultaneously a substantial depletion of contaminating neoplastic cells. Methods: We describe the results of twenty-four immunomagnetic selection procedures performed with the Isolox 300i magnetic cell separator system (Baxter, U.S.A.): 9 multiple myelomas, 12 non-Hodgkin's lymphomas, 2 chronic lymphocytic leukaemias, and 1 Waldenström's disease. Double selections using anti-CD19 and anti-CD20 monoclonal antibodies were done in all cases. To assess the double purging efficacy, CD19+ cells in the final (IP) and final product (FP) were analyzed by flow cytometry. Results: The average total CD34+ cells x 10^6 and CD34+ cells/kg x 10^6 were 559.61 ±224.50 and 8.33 ±22.32 in the IP and 283.36 ±110.74 and 4.05 ±1.48 in the FP respectively. Average purity was 99.65% (70.30-98.70) and the CD34+ yield was 49.45% (38.51-59.28). The medium reduction of CD 19+ cells was 4.06 logs±1.59. Thirteen patients were transplanted and the time of engraftment was similar to historical patients transplanted with unmanipulated products. Conclusions: Isolox 300i immunomagnetic device offers a more purging method for PBSC harvests in haematologic malignancies. Autologous transplantation with these products is safe and ensures a normal haematopoietic reconstitution.

751 FURTHER EXPERIENCE WITH CAMPATH-1H IN MONOCLONAL ANTIBODY FOR T-CELL DEPLETION OF PERIPHERAL MONONUCLEAR CELLS IN THE-BAG

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Unmanipulated allogeneic transplantation of haematopoietic stem cells derived from bone marrow or peripheral blood has a significant rejection rate, acute and chronic graft-versus-host disease (GVHD) and cytomegalovirus (CMV) infection (CMV). We have previously reported that T-cell depletion by exposure of bone marrow mononuclear cells to either yttrium or saponin forms of the antibody abolishes these three major complications and has a low relapse rate in acute myeloblastic leukaemia. A subsequent switch to peripheral blood improved outcome by shortening time to engraftment by about 7 days. We now update this experience using the humanised immunoglobulin (n~80) and confirm uniform engraftment with median time to 0.5x10^9/L neutrophils at 12 days and 50x10^9/L of platelets at 14 days. CMV was isolated in 6 patients (17.5%) and of these 4 (66%) died. A notable new feature is erythrodema considered to be late presenting acute GVHD without involvement of bilary endothelium or gut. These patients generally responded to topical steroids. In our first 5 unrelated donors, because of uncertainty about the efficiency of ex vivo T-cell depletion across antigen disparity, cyclosporin was given for 3 months without side effects. Furthermore, of the initial 4 related but mismatched grafts CMV occurred in 2 and mild GVHD in 2. In these nine engraftment was uniform and the time to haematopoietic reconstitution was the same as matched pairs. It concluded that this ex vivo manipulation is both practical and reliable in histocompatible sibling grafts. Additionally it is applicable to matched unrelated and minimally mismatched families donors but here it is not yet established whether cyclosporin post transplantation can be avoided.

752 ASSOCIATION OF DONOR IL-10 POLYMORPHISM GENOTYPE WITH ACUTE GVHD IN SIB-IDENTICAL ALLOT-MENT

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IL-1 receptor antagonist (IL-1ra) is the naturally occurring antagonist to both IL-1α and B. A VNTR polymorphism in intron 6 of the IL-1ra gene has been associated (allele 2) with increased IL-1ra production. We determined the genotype of this polymorphism in both donors and patients receiving HLA identical allogeneic bone marrow transplants. The association of allele 2 in the patients genotype showed no association with the occurrence of acute GVHD (aGVHD) for patients receiving either combination immunosuppressive therapy or cyclosporin monotherapy. However, for all immunosuppressive therapies possession of allele 2 in the donor genotype was shown to be significantly associated with less severe GVHD grade 0-1 (27/57), rather than with the more severe grades III-IV (21/22) p<0.0146 (Fishers exact test). In those transplants given cyclosporin monotherapy presence of allele 2 in the donor genotype was also significant; 20/44 with grade 0-II and 2/19 with grades III-IV (p=0.0065). We also tested for association between the less common allele of the IL-1α-3953 TaqI polymorphism in patient and donor genotypes with aGVHD occurrence. Allele 2 of this polymorphism has been associated with increased production of IL-1. No significant association was found between this polymorphism and severity of GVHD. These results show that donor genotype for the IL-10 polymorphism may have a protective role with respect to severity of GVHD following transplantation. This significant finding may be an additional factor for individual patient risk assessment for complications, including GVHD after bone marrow transplantation.

753 RECIPIENT INTERFERON-GAMMA & INTERLEUKIN-6 GENE POLYMORPHISMS ASSOCIATE WITH GVHD IN HLA-MATCHED SIBLING BMT. A GENOTYPIC GVHD RISK INDEX

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Pre-inflammatory cytokines including IFNy, IL-6 & TNFα are implicated in the "cytokine storm" mediating acute GVHD & other inflammatory BMT complications. The anti-inflammatory cytokine IL-10 has been linked to reduced GVHD. Polymorphism within cytokine genes is associated with functional differences in cytokine regulation & affected clinical performance in a variety of diseases. Polymorphism in the IFNy intron 1 (CAG) microsatellite repeat has been shown to correlate with in vitro IFNgamma production and acute renal transplant rejection. The IL-6-174(G/C) single nucleotide polymorphism allele C has been previously linked to lower in vitro and in vivo IL-6 production and with juvenile chronic arthritis. We examined potential association of GVHD with IFNy & IL-6 polymorphisms in a cohort of 87 cyclosporin A-treated HLA-matched sibling BMT donor/recipient pairs. Recipients homozygous for the IFNy intron 1 allele 3 had significantly more severe (grade III-IV) acute GVHD (p=0.013). Recipients homozygous for the IL-6-174 allele C had significantly less severe acute GVHD (p=0.012). We have previously demonstrated association of recipient TNFα and IL-10 gene polymorphisms with GVHD severity (Blood 52:394). Having found recipient IFNy & IL-6 genotype association with GVHD severity we have combined these findings with the previously identified TNF & IL-10 gene polymorphisms to examine the cumulative impact of these genotypes on GVHD severity. Recipient possession of more than one high-risk-associated genotype (TNF3383: IL-10 -1064 [1-2-16]) was associated recipient IFNy intron 1.33 correlates strongly with severe acute GVHD, even in the presence of the low-risk-associated genotype IL-6-174 CC (p<0.0003). Absence of the three recipient high-risk-associated genotypes is associated with no or mild (grade I) GVHD only (p=0.0143). We propose that a combined cytokine genotypic risk factor index may facilitate acute GVHD prediction, enabling accurate pre-BMT assessment and appropriate individual adjustment of GVHD prophylaxis.
CHAPTER 6

PERIPHERAL BLOOD STEM CELL GRAFTING

- TRANSFER OF EVOLVING TECHNOLOGY -

THE THIRD LOCAL INNOVATION
Four sets of experimental and laboratory data have combined to advance this technology. Here aspirated bone marrow rich blood becomes the source of the stem cell graft from which is recovered the matching population that is normally and predominantly resident in immunohaematopoietic niches in the medullary cavities.

Firstly the long established demonstration of the nomadic nature exhibited by these progenitor cells as having the equivalent capacity to not only repopulate empty bone marrow space in a lethal irradiation animal model but, concurrently, producing matching all lineage haematopoiesis in the spleen.\textsuperscript{177-179}

Secondly in long-term bone marrow culture to show that in blood and marrow colonies and clusters were comparable from both populations. This confirmed that a circulating pool existed and theoretically could be tapped into.\textsuperscript{180-182}

Thirdly preliminary reports translated the early experimental haematology observations into clinical reality by use of continuous flow blood fraction separators. Here a population could be identified, recovered and then shown to replicate all the essential characteristics required for successful reconstitution by engraftment of haematopoietic and immunologic lineages.\textsuperscript{168,183-185}

Fourthly was the observation, relevant in clinical context, that in a variety of animals, including non-human primates, the acquired donor material could be quantitatively and qualitatively enhanced in a range of circumstances. These manipulations are described as mobilisation and release, by altering states of adhesion via a range of different molecules pluripotential cells into blood for harvesting by mechanical means.\textsuperscript{186-188} In parallel by interacting with transfusion, immunology and transplantation communities worldwide each of these steps was established and standardised. Crucially to operate with rigorous quality control according to international standards, exemplified by the Joint Accreditation Committee of the International Society for Cellular Therapy (JACIE)\textsuperscript{189,190} and US Based Foundation for Accreditation of Cellular Therapy (FACT), to match locally approved bone marrow transplantation programs to these plans for harmonisation across countries.\textsuperscript{189}
Over the last 10 years the necessary quality control has been embodied in standard operating procedures. Accordingly after introduction and ongoing evaluation with upgrading, this somewhat rigid approach, guaranteed the consistent recovery of quantitatively and qualitatively adequate stem and progenitor cells for the clinical program\textsuperscript{4}. This mandated control, which is documented and monitored is currently, as elsewhere in the world, regarded as standard of practice\textsuperscript{10,13,52,191,192} and included a number of diseases.

Some variations exist when the specified limits for recovery of mononuclear cell numbers and CD34 expressing numbers appear inadequate\textsuperscript{4,193}. Additionally, particularly with matched-unrelated programs flexibility is crucial. For example with international collaboration through the local and corresponding registries' worldwide there persist the specified prescription that bone marrow as opposed to peripheral blood is required in some geographical areas\textsuperscript{194,195}. In this bi-directional collaboration handling of the two different graft sources needed to be accommodated and separate sets of criteria maintained\textsuperscript{196-198}.

In an extension of this ever evolving upgrading of peripheral blood stem cell grafts is the use of umbilical blood or cord donations\textsuperscript{193,199-201} including our association with Eurocord and Netcord\textsuperscript{202-204}. Additionally new studies are turning to the use of amniotic fluid\textsuperscript{205-207} and occasionally foetal liver although this is a less well-established clinical practice\textsuperscript{208,209}. Endorsement for this now firmly introduced technology underlies confirmed accreditation as a transplantation, harvest and donor centre specifically by the European Group for Blood and Marrow Transplantation\textsuperscript{10,210} and the American National Donor Program\textsuperscript{211,212}. 

\textsuperscript{10,210}
Distribution of marrow repopulating cells between bone marrow and spleen early after transplantation

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Whether hematopoietic stem cells (HSCs) home selectively to bone marrow (BM) early after transplantation remains an issue of debate. Better understanding of homing mechanisms may benefit BM transplantation protocols in cases of limited graft cell number or nonmyeloablative conditioning regimens. Using flow cytometry and serial transplantation to stringently identify HSCs, trafficking patterns of long-term engrafting cells were mapped between BM and spleen early after transplantation. Low-density BM cells were tracked in irradiated or nonirradiated mice 1, 3, 6, and 20 hours after transplantation, at which time recipient BM and spleen were analyzed for recovery of primitive donor cells by phenotype and adhesion molecule expression. In addition, phenotypically defined HSC-enriched or HSC-depleted grafts were tracked 20 hours after transplantation in recipient BM and spleen and analyzed for recovery and long-term repopulating potential in mice undergoing serial transplantation. Regardless of irradiation status, recovery of donor Sca-1+ Lin- cells was higher at most time points in recipient BM than in spleen, while recovery of total Sca-1+ cells was variable. A significantly higher percentage of BM-homed donor Sca-1+ cells expressed CD43, CD49e, and CD49d 20 hours after transplantation than spleen-homed cells, which contained significantly more non-HSC phenotypes. Furthermore, BM-homed cells were significantly enriched for cells capable of secondary multilineage hematopoiesis in mice undergoing serial transplantation compared with spleen-homed cells. These results support the notion of specific homing of HSCs to BM by 20 hours after transplantation and provide a basis for the enhanced engraftment potential afforded some Sca-1+ Lin- cells subfractionated on the basis of adhesion molecule expression. (Blood. 2003;102:2285-2291)

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Introduction

Engraftment and reconstitution of normal hematopoiesis following transplantation of hematopoietic stem cells (HSCs) rely on the ability of these cells to lodge within specialized bone marrow niches where the process of proliferation and self-renewal of stem cell functions commence for lifelong hematopoiesis. The ability to manipulate this process may hold clinical benefits in cases of limited graft cell number or compromised homing ability of ex vivo-manipulated cells. However, whether transplanted cells home to bone marrow (BM) by a coordinated series of events based on their potential or whether homing represents a nonspecific process whereby these cells are retained in BM based on the relative size of this organ remains undefined. Evidence exists to support both specific and nonspecific lodging of transplanted HSCs in BM, and data are beginning to suggest that homing mechanisms may be more functional in nonirradiated hosts. Knowledge of the specificity and kinetics of homing is an essential first step in the design of methods aimed at increasing the efficiency of transplantation.

Results from homing studies that tracked unfractonated cells or progenitors in recipient BM or spleen may not signify the trafficking of target stem cell populations. More direct homing assays have focused on tracking phenotypically defined populations of transplanted cells in host tissues by flow cytometry, or transplanting prospectively isolated purified stem cell grafts. To directly examine HSC trafficking, investigators have more recently focused on isolation and retransplantation of tracked BM- or spleen-homed cells in long-term reconstitution assays, given that hematopoietic reconstitution is regarded as the only true test of HSC function. These studies have documented the presence of repopulating cells in both BM and spleen hours after transplantation but exclusive absence in BM 48 hours later. In these studies, repopulating cells present in spleen 3 hours after transplantation provided more rapid reconstitution than BM-homed cells, suggesting the possibility of hierarchic differences in the hematopoietic potential of BM-homed versus spleen-homed cells. Functional studies of BM- versus spleen-homed cells in serial transplantation studies may more precisely define kinetics of BM homing and trafficking patterns of HSCs.

In the current investigation, trafficking patterns of transplanted stem cell grafts were determined in recipient BM and spleen using several different approaches. In the first, grafts of low-density BM (LDBM) were tracked 1 to 20 hours after transplantation by phenotypic characterization of BM- and spleen-homed donor Sca-1+ cells possessing a complex phenotype of adhesion molecules and other markers associated with repopulating cells. In the second approach, HSC-enriched and HSC-depleted grafts were tracked in vivo. Non-HSC grafts were significantly enriched in spleen tissue, while BM recovery of either group of cells was similar. However, serial transplantation studies revealed the exclusive

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presence of long-term repopulating cells in BM rather than spleen by 20 hours after transplantation. Collectively, these studies support the notion of selective homing or survival of HSCs in BM 20 hours after transplantation.

Materials and methods

**Mice**

C57BL/6 mice (CD45.2 allele) were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 to 10 weeks of age and allowed to acclimate for 1 to 2 weeks prior to use in their studies. B6.5L/Per.CpFe/5Boyl (B6.Boyl) congenic mice (CD45.1 allele) were either purchased from Jackson Laboratories or maintained in our breeding colony and used between 9 and 12 weeks of age. All studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

**Primary short-term (1ST) cell tracking**

BM grafts consisted of LDMB cells or purified Sca-1- lin- cells subfractionated on the basis of CD49e or CD262 expression into fractions enriched for long-term engraftment potential (ENG: Sca-1- lin- CD49e+ or Sca-1- lin- CD262L+ or those devoid of such potential (non-ENG: Sca-1- lin- CD49e- or Sca-1- lin- CD262L-)) as previously documented. Adhesion molecules CD49e and CD262L were chosen in these studies so that grafts would consist of phenotypes where both positive expression and negative expression enriches for long-term repopulating cells (CD49e+ and CD262L+) and nonrepopulating cells (CD49e- and CD262L-), as previously shown. Donor LDMB, ENG, and non-ENG cells were isolated from C57BL/6 or B6.Boyl mice and tracked in vivo with PKH26 or PKH2 (Sigma, Immunocytometry Systems, San Jose, CA) and 1 x 10⁴ to 5 x 10⁴ events were acquired per sample. BM, spleen, and PB from 1ST recipients of ENG and non-ENG transplants were analyzed for presence of donor cells by flow cytometric analysis of data files containing 2 x 10⁴ to 1 x 10⁵ events. No further phenotyping was performed in ENK/non-ENG experiments. In all experiments, donor cells were distinguished from recipient by PKH26, PKH26, CFSE, or appropriate CD45.1 or CD45.2 staining as described in "Primary short-term (1ST) cell tracking."

**Calculation of recovery of primitive donor cells**

The frequency of donor cells falling within a light scatter gate including lymphocytes and large granular cells was determined for each harvested tissue based on the background fluorescence of cells from control mice, as previously described. Control mice were subjected to the same treatment at experimental groups, including radiation and injection of vehicle. Frequency of Sca-1+ cells among donor cells and frequency of lin- cells among donor Sca-1+ cells were likewise determined and used in conjunction with tissue cellularity to calculate recovery of primitive phenotypes after LDMB transplants. Frequencies were multiplied by the total number of cells in each tissue and then divided by the number of cells in the original graft to calculate the recovery of transplanted cells. The number of BM cells harvested from both thymi and femurs was considered to represent 18.7% of total marrow volume. The total volume of PB was assumed to be 2 mL per mouse. A sample calculation of recovery of Sca-1+ cells in nonirradiated BM is as follows: No. BM cells in whole mouse (210 x 10⁶) x Frequency of donor cells in BM (2%) = No. BM-bred donor cells (4.2 x 10⁷) x Frequency of Sca-1+ cells among donor cells (20%) = No. BM-bred Sca-1+ donor cells (0.84 x 10⁷)/No. Sca-1+ cells injected (6.4 x 10⁶) = 0.13 x 10⁴ = 1.6% Recovery of Sca-1+ donor cells in BM (13%).

Similar calculations were then used to obtain the recovery of lin- Sca-1+ donor cells in BM. Due to the low frequency of detectable donor cells when ENK or non-ENG cells were injected, the frequency of donor cells was calculated manually using event count of saved fluorescence-activated cell sorted (FACS) files.

**Serial transplantation studies**

BM- and spleen-bred donor cells from irradiated or nonirradiated 1ST recipients of ENG cells were isolated 20 hours after transplantation by flow cytometric cell sorting using CD45.1,41-P and CD45.2-fluorescein isothiocyanate (CD45.2-FITC). Identical numbers of sorted donor cells (range, 50-500 cells per mouse), along with 1 x 10⁴ competitor LDMB cells of recipient origin, were transplanted into primary long-term (1LT) congenic recipients within 3 hours of receiving lethal irradiation in a split dose of 700 cGy plus 350 cGy 4 hours apart. Recipient mice were bled from the tail vein monthly until 6 months for analysis of donor-derived hematopoiesis. In 3 experiments (1 irradiated 1ST and 2 nonirradiated 1ST), 1LT recipients were killed 6 months after transplantation, and 1 x 10⁴ to 5 x 10⁵ LDBM cells were transplanted into lethally irradiated (700 cGy plus 350 cGy) secondary L1 (2LT) recipients without competition. 2LT recipients were bled from the tail vein monthly until 6 months for analysis of donor-derived hematopoiesis. Donor-derived cells from some 1LT and 2LT L cells were analyzed 6 months after transplantation for the percentage of lineage cells using PE-conjugated antibodies specific for CD3+, B220+, Gr-1, and Mac-1 cells.

**Statistical analysis**

Data are expressed as the mean ± SEM where applicable. Differences between groups were analyzed using an unpaired 2-sided t test. Differences in chimerism were analyzed by repeated measures analysis of variance using an arcsine transformation. A probability value of less than .05 was considered significant for all tests.
Results

Primitive phenotype of BM- and spleen-homed donor cells

To investigate trafficking patterns of primitive hematopoietic progenitor cells (HPCs), irradiated or nonirradiated mice underwent transplantation with LDBM and were killed 1, 3, 6, or 20 hours later. Donor cells harvested from BM or spleen or circulating in PB were phenotyped for expression of primitive markers. In irradiated mice, the frequency of Sca-1+ donor cells in BM and spleen was similar and 3 hours after transplantation but increased significantly in spleen by 20 hours (Figure 1A). The frequency of Sca-1+ donor cells in nonirradiated mice was significantly higher in spleen than BM at 3, 6, and 20 hours after transplantation (Figure 1C). Cellularity of BM, spleen, and PB from irradiated mice ranged from 24 × 10^6 to 55 × 10^6, 33 × 10^6 to 88 × 10^6, and 1 × 10^9 to 4 × 10^9, respectively, depending on the time after irradiation that mice were killed. Mean cellularity of BM, spleen, and PB from nonirradiated mice was 212 × 10^6, 72 × 10^6, and 14 × 10^6, respectively. Data on cellularity are reproduced from Peltt and colleagues. Due to the higher cellularity of BM compared with spleen, recoveries of Sca-1+ cells did not differ significantly between BM and spleen in either transplantation group (Figure 1B, D).

In contrast to total Sca-1+ cells, frequencies and recoveries of the more primitive Sca-1+ lin-1 donor cells in irradiated and nonirradiated recipients tended to be higher in BM than spleen, although these data were not significant at all time points (Figure 2).

Adhesion molecule phenotype of BM- versus spleen-homed donor cells

We previously examined the expression of adhesion molecules on BM-homed Sca-1+ donor cells 1 to 20 hours after transplantation in irradiated or nonirradiated recipients. In the present study, we compared concurrently the adhesion molecule expression of BM- and spleen-homed cells in irradiated recipients. Expression of CD43, CD49e, and CD49d significantly decreased on spleen-homed Sca-1+ cells between 1 and 20 hours after transplantation, while remaining relatively constant on BM-homed Sca-1+ cells (Figure 3A-C). By 20 hours after transplantation, expression of these 3 adhesion molecules, previously shown to be present on primitive HPCs from both murine and human sources, was significantly greater on BM-homed Sca-1+ cells than on similar spleen-homed cells (Figure 3A-C). Because the cellularity of BM was higher than that of spleen, not only was the frequency of CD49e+, CD43-, and CD49d- Sca-1+ cells higher in BM than in spleen, but absolute numbers were also higher in BM (4.3, 2.8, and 1.7-fold higher at 20 hours after transplantation, respectively). Similar results were observed in nonirradiated mice (data not shown).

The frequency of CD62L- cells increased among BM- and spleen-homed Sca-1+ cells until approximately 70% of these cells expressed CD62L by 20 hours (Figure 3D). Expression of CD41 and CD44 was not different between BM- or spleen-homed Sca-1+ cells at any time point and ranged from 95% to 100% on donor Sca-1+ cells in all tissues examined (n = 4, data not shown).

Differential trafficking of ENG versus non-ENG cells in irradiated mice

We next examined the in vivo movement of BM cells highly enriched for long-term engraftment potential in comparison to BM cells known to be devoid of such activity. Such a strategy would identify whether long-term engrafting cells possess specific trafficking mechanisms and whether nonengrafting cells lack such mechanisms, which may in turn contribute to their lack of stem cell function. Based on our previously published work identifying cells with long-term engrafting potential, purified populations of engrafting cells (ENG) consisting of Sca-1+ lin- CD49e+ or Sca-1+ lin- CD49d- cells of nonengrafting cells (non-ENG) containing Sca-1+ lin- CD49e- or Sca-1+ lin- CD49d+ cells were...
isolated prior to transplantation and tracked in vivo. Irradiated 
1ST mice received $4 \times 10^4$ to $3 \times 10^5$ sorted ENG or non-ENG 
cells, and then BM, spleen, and PB were analyzed 20 hours later for 
the presence of donor cells by flow cytometric analysis. Frequency 
and recovery of donor cells did not differ between non-ENG and 
ENG grafts in recipient BM (recovery = 4.3% ± 1.4% and 
4.2% ± 1.4%, respectively; n = 10-11). However, frequency of 
non-ENG cells was consistently higher in spleen than that of ENG.

Figure 3. Frequency of CD43+ , CD44+ , CD49d+, and CD92L+ 
cells among donor Sca-1+ cells in BM, spleen, and PB at 1, 3, 6, and 26 
hours following transplantation of low-density BM cells. Mice were 
irradiated with 900 cGy, underwent transplantation an average of 18.5 
hours later with $1 \times 10^5$ to $1 \times 10^6$ LDBM cells, were killed at the indicated 
time points, and donor Sca-1+ cells in BM and spleen were analyzed for 
expression of CD43 (A), CD44d (B), CD49d (C), and CD92L (D) by flow 
cytometry as described in "Materials and methods." All 4 time points were 
assayed in every experiment. Data are expressed as the mean ± SEM 
percent of donor Sca-1+ cells with light scatter properties characteristic of 
primitive hematopoietic cells and that express each adhesion molecule. 
n = 4. *P < 0.05 when compared with spleen at the same time points, 
**P < 0.05 when compared with earlier time points of same tissue. Data for 
BM cells are partially reproduced, with permission, from Piwek et al.

Figure 2. Frequency and recovery of lineage-negative cells among 
Sca-1+ donor cells in BM, spleen, and PB for times up to 26 hours following 
transplantation of low-density BM cells in irradiated and nonirradiated 
recipients. Mice underwent transplantation with $1 \times 10^7$ 
to $1 \times 10^6$ LDBM cells, were killed at the indicated time 
points, and were analyzed for frequency and recovery of donor 
Sca-1+ lin- cells in BM, spleen, and PB as described in "Materials and methods." Data in panels A and 
C are expressed as the mean ± SEM percent of 
donor Sca-1+ cells falling within a light scatter gate 
including lymphocytes and large granular cells and that 
lack lineage expression. Horizontal lines in panels A and 
C represent the mean percentage of lineage-negative 
donor Sca-1+ cells in the original graft. Using a similar 
light scatter gate, recoveries of Sca-1+ lin- donor cells 
were calculated and are shown in panels B and D. Bars in 
panels B and D represent mean ± SEM recovery of 
Sca-1+ lin- donor cells (sum of recoveries in BM plus 
spleen plus PB), and lines represent mean ± SEM 
recoveries in BM, spleen, or PB. Numbers above each 
bar represent mean recoveries. n = 6-12 in panel A, 
n = 4-11 in panel B; n = 4-5 in panel C; n = 5-6 in panel D. 
*P < 0.05 when compared with spleen of the same time 
point. To provide an SEM value for total recovery at each 
time point, total recoveries were not calculated by adding 
the means of the recoveries from each tissue but rather 
by averaging the total recoveries from each individual 
experiment.
undergoing serial transplantation. To this end, 50 to 200 BM- or spleen-homed donor cells were isolated from irradiated or nonirradiated 1ST recipients 20 hours after transplantation and equal numbers transplanted competitively into 1LT irradiated recipients. In 8 experiments using a total of 41 mice, no differences were noted in the degree of donor-derived chimerism in 1LT mice up to 6 months after transplantation of BM-homed or spleen-homed cells, regardless of irradiation status of 1ST recipients (Figure 5A, pooled data from irradiated and nonirradiated 1ST mice). Six months later, 1 x 10^6 to 5 x 10^6 LDBM cells from 1LT mice in 3 experiments were transplanted into 2LT irradiated mice without competition. Interestingly, chimerism was significantly higher in mice that received cells initially homed to BM rather than spleen in 1ST recipients (Figure 5B, pooled data from 1 experiment with an irradiated 1ST recipient and 2 experiments with nonirradiated 1ST recipients). In one particular experiment where spleen-homed cells provided 88% donor-derived hematopoiesis in 1ST recipient BM, 70% of 2LT recipients undergoing transplantation with this marrow failed to survive beyond 1 month of transplantation, illustrating the inability of spleen-homed cells to support hematopoiesis in secondary recipients.

No differences in chimerism in 1LT or 2LT mice were noted when Sca-1+/lin- CD49e+ or Sca-1+ lin- CD42L+ cells were used.
in the initial homing step in 1ST mice. In addition, BM-homed and spleen-homed cells from 1ST mice did not apparently differ in their relative contribution to either myeloid or lymphoid lineages (data not shown).

Discussion

In this report, evidence is presented that supports the notion of selective distribution or homing of transplanted HSCs to BM during the first 20 hours after transplantation. Phenotypic evidence documenting increased frequency and recovery of donor Sca-1+ lin- cells in BM relative to spleen, as well as increased frequency and recovery in BM of adhesion molecule phenotypes known to be enriched for HSCs, further support this notion. Significant enrichment of purified non-ENG grafts in spleen 20 hours after transplantation suggests trapping of nonengrafting cells in secondary hematopoietic tissues. Finally, selective homing of HSCs to BM is further supported by serial transplantation studies that revealed the exclusive presence of cells capable of secondary long-term multilineage hematopoiesis in BM by 20 hours after transplantation, while donor cells in spleen at this time failed to provide secondary donor-derived hematopoiesis and, in some cases, failed to provide radioprotective support in 2LT recipients.

Previous reports have documented the presence of rapid reconstituting ability in spleen-homed donor cells collected 3 hours after transplantation but exclusive presence of repopulating cells among BM-homed cells by 48 hours.1 In the present studies, both BM- and spleen-homed donor cells isolated 20 hours after transplantation from both irradiated and nonirradiated 1ST recipients were capable of supporting hematopoiesis in 1LT recipients to a similar degree, but BM-homed cells provided a significantly higher degree of chimerism in 2LT recipients than spleen-homed cells. Of interest was the apparent lack of radioprotective cells in the marrow of some 1LT recipients of spleen-homed cells, despite high levels (up to 85%) of donor-derived chimerism. These results suggest that 20-hour spleen-homed cells, while capable of sustaining long-term hematopoiesis for one generation, do not contain the most primitive HPCs capable of providing hematopoiesis in secondary recipients and illustrate a hierarchic process that results in efficient homing of the most primitive HPCs to BM rather than to spleen during the first 20 hours following transplantation. These results corroborate those of Szilvassy et al,5 in which this group reported more rapid reconstitution by 3-hour spleen-homed cells than BM-homed, emphasizing the degree of heterogeneity in the stem cell pool and possible presence of more "mature" HSCs in spleen at this time point. The relatively higher recovery in spleen of total Sca-1+ cells (Figure 1) compared with the more primitive lin- fraction that was relatively enriched in BM, especially in nonirradiated mice (Figure 2), further supports the notion that more mature cells home to spleen. Alternatively, it is also possible that transplanted HSCs survive better in BM than spleen during the first 20 hours after transplantation as previously suggested,10 contributing to their engraftment potential.

To eliminate variables influencing stem cell phenotype after transplantation, and to test the possibility that certain classes of cells fail to engraft due to an inability to efficiently home to the BM, we examined the homing of populations of cells highly enriched for HSCs (ENG) and those lacking this potential (non-ENG). Notably, although ENG cells were highly enriched for HSCs,10 this phenotype was still heterogeneous in stem cell potential and in vivo trafficking ability, as revealed in selective homing of long-term repopulating cells to BM while cells of lesser potential segregated to spleen. Homing experiments performed in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice suggest little selectivity in BM-versus spleen-homing of transplanted primitive HPCs.11-12 Whether differences in results obtained in NOD/SCID mice and the present studies are secondary to xenogeneic homing models or differences in stringency of HSC assays requires further investigation.

Interestingly, trafficking of ENG and non-ENG cells to BM was similar, suggesting that defects in homing are not the cause of the reported engraftment failure of non-ENG cells but, rather, limited intrinsic hematopoietic potential. Whether differences in adhesion molecule expression between ENG and non-ENG cells translates to differences in the ability of these 2 groups of cells to communicate effectively with the BM microenvironment for determination of their fate remains an interesting possibility that may explain lack of engraftment potential of non-ENG cells. While several adhesion molecules have been implicated at various steps of homing and egress from the BM,13-15 a direct role for any adhesion molecule in homing of HSCs has not been documented. Because interactions between CD49d and fibronectin are known to stimulate proliferation and differentiation of primitive HPCs (reviewed by Chun and Watt),16 it is likely that cells lacking CD49d expression are unable to effectively contribute to BM hematopoiesis. A role for CD62L in hematopoiesis is less well defined, but data are beginning to suggest that this molecule may be more important for progenitor cell function than stem cell function,9,26,31,32 providing a possible reason for the rapid up-regulation of CD62L on BM-homed Sca-1+ cells in the present studies. We and others have previously documented the presence of CD49d+ and absence of CD49d- on murine long-term engrafting cells. We now show that lack of expression of CD49d and expression of CD62L predisposes Sca-1+ lin- cells to sequestration in the spleen, suggesting the direct involvement of these or other, yet unknown, adhesion molecules in directing the trafficking of hematopoietic cells. The percentage of non-ENG cells recovered in spleen was nearly twice that of ENG cells and similar to that previously reported for grafts of LDBM (1.5% ± 0.2%), another source of cells composed largely of nonengrafting cells. Our present studies provide a mechanistic link between phenotypically defined engrafting cells identified previously by our group based on expression of adhesion molecules and the potential of these molecules to direct the homing of engrafting cells to the BM.

Increased frequency and recovery of CD43+, CD49e+, and CD49d+ Sca-1+ donor cells in BM compared with spleen was greatest 20 hours after transplantation. These results can potentially be explained by either trafficking of these phenotypes from spleen to BM between 1 and 20 hours, as previously suggested,5,9 or modulation of the expression of these markers on Sca-1+ cells that had already homed to BM or spleen in the first 20 hours (possibly to facilitate anchorage of these cells). Because recovery of total Sca-1+ cells remained fairly constant in spleen while the percentage of CD43+, CD49e+, and CD49d+ Sca-1+ cells declined, and because recovery of ENG cells in BM was similar between 3 and 20 hours (4.0% ± 1.3% and 4.2% ± 1.4%, respectively), it is plausible that phenotypic down-modulation of adhesion markers on spleen-homed cells is responsible for the observed changes. However, trafficking of donor cells between BM and spleen during this time frame cannot be ruled out. It is also possible that BM and spleen act cooperatively to ensure efficient homing of transplanted HSCs to BM; in this case spleen would be a necessary component of the homing process. Manipulation of the trafficking of transplanted HSCs by blocking pathways involved in spleen homing, or via splenectomy, may provide more precise answers to the role of spleen in homing of primitive HPCs to BM.
We have previously documented an enrichment of long-term repopulating cells in BM of nonirradiated recipients compared with irradiated ones. This observation may signify a more hospitable environment and more efficient trafficking of cells to nonirradiated BM compared with ablated BM, where endothelial damage may hinder efficient movement of cells to BM and/or increase nonspecific binding of cells to other sites. Our present studies add to these results by showing that even within an irradiated host, despite endothelial damage and relatively inefficient trafficking, a certain degree of selective trafficking to BM remains. It is possible that host influences elaborated by irradiated tissues promote proliferation and survival of transplanted cells in BM. Whether the differing or timing of host conditioning prior to transplantation can be manipulated to better preserve a microenvironment that is more conducive for homing warrants further investigation. Nevertheless, these results begin to define a basis for the enhanced engraftment potential afforded some groups of Sca-1+ LSA cells fractionated on adhesion molecule expression and begin to outline distinct in vivo trafficking patterns of transplanted long-term engrafting cells within the first 20 hours of transplantation.

References


In vivo trafficking, cell cycle activity, and engraftment potential of phenotypically defined primitive hematopoietic cells after transplantation into irradiated or nonirradiated recipients

P. Artur Plett, Stacy M. Frankovitz, and Chrisle M. Orschell-Traycoff

Recent interest in bone marrow (BM) transplantation in nonconditioned or minimally conditioned recipients warrants investigation of homing patterns of transplanted hematopoietic progenitor cells (HPCs) in irradiated and nonirradiated recipients. To this end, phenotypically defined populations of BM cells were tracked in lethally irradiated or nonirradiated mice at 1, 3, 6, and 24 hours after transplantation. Recovery of transplanted cells at all time points was higher in BM of nonirradiated mice, similar to earlier suggestions. The percentage of lineage-negative Sca-1+ cells and Sca-1+ cells expressing CD43, CD49e, and CD49d steadily increased in BM of nonirradiated mice up to 24 hours, while fluctuating in irradiated mice. Cell cycle status and BrdU incorporation revealed that less than 20% of Sca-1+ cells and fewer Sca-1+Lin+ cells had cycled by 24 hours after transplantation. To more directly examine trafficking of primitive HPCs, purified grafts of CD62L+ or CD49e+ subfractions of Sca-1+Lin- cells, previously shown to be enriched for long-term repopulating cells, were also tracked in vivo. Recovery of purified cells was similarly increased in BM of nonirradiated mice. When 50 to 100 of these BM-homed cells were examined in serial transplantation studies, BM-homed cells from initially nonirradiated mice were enriched 5- to 30-fold for cells capable of long-term hematopoiesis in secondary recipients. Collectively, these data suggest that homing or survival of transplanted cells in irradiated recipients is less efficient than that in nonirradiated recipients, implicating an active role of radiation-sensitive microenvironmental cues in the homing process. These results may have important clinical implications in the design of BM transplantation protocols. (BLOOD. 2002;100:3545-3552)

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Homing, engraftment, and fate of transplanted hematopoietic stem cells (HSCs) remain poorly understood phenomena. While some studies support the specificity of certain components of the homing process, such as chemotaxis, extravasation, anchorage, survival, and proliferation of transplanted HSCs, other studies show no differences in the seeding efficiencies of mature and primitive bone marrow (BM) stem cell subsets or in the distribution of BM-homed cells in secondary recipients, suggesting that homing is not specific.

The majority of in vivo tracking studies have focused on examining the trafficking and cycling activity of phenotypically undefined populations of BM cells, with little regard for phenotypic characterization of donor cells homing to specific organs. It is possible that HSCs, which constitute less than 1% of graft cells, may proficiently home to BM, but that their specific homing is concealed by the massive movement of the large cohort of other, more mature BM cells. Studies examining homing of colony-forming cells may not accurately reflect the homing of long-term repopulating cells. Examinations of populations of cells defined on the basis of stem cell phenotype are beginning to show a different, possibly more specific, picture of homing. Identification of the adhesion molecule repertoire of primitive hematopoietic progenitor cells (HPCs) homing to the BM shortly after transplantation may provide evidence of adhesion molecules potentially involved in trafficking, homing, and lodging of transplanted HSCs in the BM and may outline a sequential requirement of different adhesion molecules at different stages of homing.

Investigations into the homing of HSCs following transplantation have most commonly been performed in myeloablative recipients. Increased vascular permeability resulting from radiation damage of endothelial and stromal cells may lead to nonspecific seeding of transplanted cells in various organs based largely on tissue mass and vascularity. Studies showing broader tissue distribution of donor cells in irradiated recipients and greater recovery of donor cells in BM of nonmyeloablative murine recipients suggest more efficient homing or better survival of primitive HPCs in nonmyeloablative marrow. Homing patterns of primitive HPCs in nonmyeloablative recipients may be even less informative, as nonmyeloablative recipients may lack the radiation-induced tissue damage and inflammation that can influence the homing patterns of transplanted HPCs. Studies using conditional models of nonmyeloablative or minimally myeloablative recipients, in the present work, trafficking patterns of primitive HPCs were mapped in the first 24 hours after transplantation of grafts.
consisting of either unfractionated low-density BM cells or purified Sca-1+ lineage (Lin-) fractions. We examined the in vivo distribution, recovery, cell cycle status, adhesion molecule expression patterns, and secondary long-term engraftment potential of cells homing to the BM of irradiated or nonirradiated mice. The results presented herein demonstrate that transplanted HSCs capable of long-term secondary hematopoiesis home more efficiently or survive better in nonirradiated recipients, which may have important clinical implications for BM transplantation in nonablated or minimally ablated patients.

Materials and methods

Mice

C57BL/6 female mice (CD45.2 allele) were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 to 10 weeks of age and allowed to acclimate for 1 to 3 weeks prior to their use in these studies. B6.SJL-Pgk-1<sup>−/−</sup>B6.129.S2-Pkk2<sup>−/−</sup> (86.Boyl) congenic mice (CD45.1 allele) were either purchased from Jackson Laboratories or maintained in our breeding colony and used between 8 to 12 weeks of age. All studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Donor cells

Bone marrow grafts consisted of low-density BM (LDBM) cells or purified Sca-1<sup>−</sup> lineage (Lin<sup>−</sup>) cells subfractionated on the basis of CD49e or CD26L expression into fractions enriched for long-term engraftment potential ([ENG]), Sca-1<sup>−</sup> lineage (Lin<sup>−</sup>CD26L<sup>−</sup>) as previously described. Adhesion molecules CD49e and CD26L were chosen in these studies so that grafts would consist of phenotypes where both positive expression (CD49e<sup>+</sup>) and negative expression (CD26L<sup>−</sup>) are enriched for long-term repopulating cells, as previously described. Donor LDBM or ENG cells were isolated from C57BL/6 or 86.Boyl mice and stained with either PKH26 or PKH6 (Sigma ImmunoChemicals, St. Louis, MO) as previously described, or with 0.5-1.0 μM CFSE<sup>−</sup> (Molecular Probes, Eugene, OR) according to manufacturer's instructions. After staining, cells were washed extensively and resuspended in complete medium (CM) (RPMI modified Dulbecco medium [RPMI] supplemented with 10% fetal calf serum [FCS]; Hyclone, Logan, UT), 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). All of the CM ingredients (except for FCS) were obtained from BioWhittaker (Walkersville, MD). When available, congenic donor cells were not labeled with any dye and were identified by appropriate CD45.1 or CD45.2 expression by flow cytometry. The choice of the green fluorescent dye CFSE and PKH26 or the red fluorescent dye PKH6 was based on the nature of the analyte to be performed on the cells after staining. The different tracking methods used in different experiments were evenly distributed along the time course of analyses after transplantation and were equally effective in detecting donor cells. Since CFSE<sup>−</sup>-stained cells exhibit aberrant cell surface staining of some antigens for 1 to 2 hours following CFSE<sup>−</sup>-staining (C.F.O.-T., unpublished data, 2000), cells were not tracked with CFSE<sup>−</sup> at the 1- and 3-hour time points.

Primary short-term (1<sup>st</sup>ST) cell tracking

Female recipient C57BL/6 or 86.Boyl mice between 10 and 12 weeks of age were lethally irradiated with 950 cGy administered in a single dose from a 137 Cs gamma irradiated (GammaCell 40; Nordion International, Kanata, ON, Canada) 17.5 to 20 hours prior to transplantation (mean ± 18.5 hours). Irradiated or nonirradiated mice received transplants via tail vein injections of 1 × 10<sup>5</sup> to 1 × 10<sup>6</sup> LDBM donor cells or 4 × 10<sup>5</sup> to 4 × 10<sup>6</sup> purified ENG cells. In experiments where BM-homed ENG cells were isolated and assayed for long-term engraftment potential, (<sup>1</sup>ST)-irradiated recipients were injected with at least 1 × 10<sup>5</sup> graft cells, while nonirradiated mice received at least 2 × 10<sup>5</sup> purified cells to facilitate donor cell isolation. Mice were killed 1, 3, 6, and 20 to 24 hours later, and BM, spleen, peripheral blood (PB), lung, and liver were collected and single-cell suspensions prepared and lysed. Lysed cells from LDBM transplant were analyzed for donor cell recovery, primitive phenotype, adhesion molecule expression, cell cycle status, and proliferation history, while lysed cells from ENG transplant were analyzed for donor cell recovery and engraftment potential in serial transplantations.

Calculation of recovery of donor cells

The frequency of donor cells falling within a light scatter gate including lymphocytes and large granular cells, was determined for each harvested tissue, based on the background fluorescence of cells from unmanipulated mice. This frequency was multiplied by the total number of cells in each tissue, then divided by the number of cells in the original graft to calculate the recovery of total transplanted cells. The number of BM cells harvested from both tibiae and femurs was considered to represent 18.7% of total marrow, or 40% if humerus and pelvic bones were also included. The total number of PB cells was calculated assuming the total PB volume to be 2 ml per mouse. Because of the low frequency of detectable donor cells when ENG cells were injected, flow cytometric files containing 2 × 10<sup>6</sup> to 1 × 10<sup>7</sup> events were saved and the frequency of donor cells calculated manually using event count.

Adhesion molecule and lineage analysis of harvested donor cells

Harvested BM cells from recipient of LDBM grafts were stained with Sca-1 and biotinylated antibodies to either CD11b (clone 2D10), CD43 (clone 5R), CD44 (clone 1H10), CD49e (clone 5H10.32; MFR5), CD31 (clone MEC 13.3), CD63 (clone MEL-14), or CD3 and CD45RB/B20. Biotinylated antibodies were developed with streptavidin-allophycocyanin (APC; Molecular Probes). Donor cells were distinguished from recipient by PKH2, PKH6, CFSE, or appropriate CD45.1 or CD45.2 staining as described above. All antibodies were from BD PharMingen (San Diego, CA). Donor cells exhibiting small light scatter properties characteristic of primitive cells were gated and examined for Sca-1 expression and primitive phenotype (CD3<sup>−</sup>CD45RB<sup>−</sup>) or adhesion molecule expression using a FACScan or FACSCalibur (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA). Between 1 × 10<sup>5</sup> and 5 × 10<sup>5</sup> events were acquired per sample. To avoid seeing very large files, in some cases only donor-positive events were saved during flow cytometric acquisition.

Serial transplantation studies

BM-homed donor cells from 1<sup>ST</sup> recipients of ENG cells were isolated by flow cytometry, and 50 to 200 of these cells, along with 1 × 10<sup>4</sup> competitor LDBM cells of recipient origin, were transplanted into primary long-term (<sup>1</sup>ST)engraftment recipients within 3 hours of receiving lethal irradiation in a split dose of 700 cGy plus 350 cGy 4 hours apart. 1<sup>ST</sup> recipients were bled from the tail vein monthly for 6 to 7 months for analysis of donor-derived hematopoiesis by determining the percentage of CD45<sup>+</sup> or CD45<sup>−</sup> PB leukocytes. In some experiments, 1<sup>ST</sup> recipients were killed 6 to 7 months after transplantation, and 2 × 10<sup>6</sup> to 1 × 10<sup>7</sup> BM cells were transplanted into lethally irradiated (700 cGy + 350 cGy) secondary LT (<sup>2</sup>ST) recipients without competitor cells. 2<sup>ST</sup> recipients were bled from the tail vein monthly for 6 to 7 months for analysis of donor-derived hematopoiesis. In some experiments, donor-derived cells from 1<sup>ST</sup> and 2<sup>ST</sup> recipients were analyzed 6 to 7 months after transplantation for the percentage of lineage cells using phycoerythrin (PE)-conjugated antibodies specific for CD<sup>3</sup>, CD<sup>45RB</sup>, Gr-<sup>1</sup>, and Mac-1 cells.

Cell cycle status

Fresh or donor-derived Sca-1<sup>−</sup> cells from harvested BM and spleen of transplant recipients were isolated by cell sorting using a FACStar<sup>+</sup> or FACSVantage S flow cytometer (BDIS) and analyzed for cell cycle position using propidium iodide (PI) as previously described. The low
number of donor Sca-1+ cells attainable from PB precluded cell cycle analysis of these cells.

BrDU administration
Mice received 4 intraperitoneal injections of 100 µg BrDU in 200 µL H2O at 26, 24, 12, and 1 hour before receiving transplants of LDBM cells. Mice destined for 24-hour homing studies received an additional injection of BrDU 12 hours after BM transplantation.

BrDU staining
Donor-derived cells from mice administered BrDU were assayed for BrDU uptake by 2 different methods. In the first, donor Sca-1+ cells from BM or spleen at 1, 3, 6, or 24 hours after transplantation were isolated by flow cytometric cell sorting and then stained for BrDU as previously described. Briefly, sorted Sca-1+ cells were fixed with 1% formaldehyde (Tobininski, Rockville, MD) and 0.2% Tween 20 (Sigma) in phosphate-buffered saline (PBS) for at least 10 minutes but no longer than 24 hours. Cells were then treated with 4 M HCl in 0.5% Tween 20/PBS for 30 minutes at 37°C, washed with 0.1 M sodium borate (Sigma), and then washed with 0.2% Tween 20/PBS. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-BrDU antibody (BD Pharmingen). Prior to acquisition, PI was added to a final concentration of 10 µg/mL for 60 minutes.

In the second method, bulk unsorted BM or spleen cells were analyzed for flow cytometrically using 4-color analysis for donor origin, surface phenotype, and BrDU incorporation simultaneously as previously described with slight modifications. Briefly, cells were stained with the appropriate anti-CD45.1-PE or anti-CD45.2-PE to identify donor cells, biotinylated Sca-1, anti-CD3 eychocyte, and anti-B220 eychocyte, followed by streptavidin-APC, then fixed with 1% formaldehyde overnight. In some experiments, Sca-1 eychocyte was used with biotinylated lineage markers developed with streptavidin-APC. All antibodies were from BD Pharmingen. Cells were permeabilized with 0.1% saponin PBS (Perm Buffer) plus 2% formaldehyde for 10 minutes at room temperature, pelleted, then permeabilized with 0.2% Tween 20/PBS for 10 minutes at room temperature, washed with Perm Buffer, and incubated with 100 to 500 Kunitz units of DNase (Sigma) in Hanks balanced salt solution for 60 minutes at 37°C. Cells were washed in Perm Buffer and then stained with anti-BrDU-FITC (BD Pharmingen).

Statistical analysis
Data are expressed as the mean ± SEM where applicable. Differences between groups were analyzed using an unpaired 2-sided t-test. Differences in chimerism were analyzed by repeated measures analysis of variance using an arcsine transformation. A probability value of less than 0.05 was considered significant for all tests.

Results
Identification of graft sizes allowing for adequate detection of homed cells
As a first step in examining the distribution of donor cells in vivo following transplantation, the number of donor cells homing to BM, spleen, or remaining in PB after transplantation was determined. Log increasing doses of donor LDBM cells from 1 × 10^6 to 1 × 10^10 cells were injected into single irradiated mice and allowed to home for 3 hours. The frequency of donor cells detected at 3 hours in all 3 tissues examined positively correlated with the number of graft cells injected (Table 1). Based on these results, subsequent experiments were designed to deliver between 17 and 90 × 10^6 cells per graft.

Recovery of donor cells in LDBM transplants
Total recovery (BM + spleen + PB) of graft cells was calculated based on the frequency of donor cells detected in BM, spleen, and PB, and the total number of nucleated cells within each tissue, as described in "Materials and methods." The frequency of donor cells ranged from 0.3% in nonirradiated spleen at 24 hours to 39% of total cells in 1-hour irradiated PB. The cellularity of irradiated BM, spleen, and PB in recipient mice ranged between 24.55 × 10^6, 3.8 × 10^6, and 1.4 × 10^7, respectively, depending on the time of analysis after transplantation (range, 19.5 hours for 1-hour time points to 42.5 hours for 20- to 24-hour time points). Cellularity of nonirradiated BM, spleen, and PB averaged 2.12 × 10^7, 7.2 × 10^6, and 1.4 × 10^7, respectively.

Total recovery of donor cells did not vary significantly between 1 and 24 hours in either transplant setting but was, however, slightly higher in nonirradiated recipients at each time point compared with irradiated mice (bars in Figure 1A-B), in agreement with previous suggestions. Total recovery in nonirradiated mice was significantly higher than that in irradiated mice when data from all 4 time points were pooled together (7.6 ± 0.6, n = 18; and 5.6 ± 0.3, n = 38, respectively, P < .05). When individual tissues were examined in both transplant settings, recovery of donor cells was highest in BM from 3 to 24 hours after transplantation (Figure 1A-B), reflecting either the specificity of BM homing of graft cells or the relatively larger mass of this tissue, or both. Distribution of donor cells differed with time in the 2 transplant settings. While recovery of donor cells in BM increased rapidly and reached a plateau by 3 hours in irradiated mice, recovery in nonirradiated marrow remained fairly constant. Recovery in nonirradiated BM was significantly greater than that in irradiated BM when data from all 4 time points were pooled together (5.7 ± 0.5%, n = 18; and 3.70% ± 0.2%, n = 38; respectively, P < .05). Interestingly, while recovery in irradiated spleen did not change significantly over time, donor cells accumulated initially in nonirradiated spleen and then declined at later time points (Figure 1A-B). As expected, cell recovery was highest in PB at 1 hour but declined thereafter. In initial experiments, lung and liver were also examined for the recovery of donor cells, but since total recovery in the 2 tissues combined was typically less than 0.1%, these tissues were omitted from subsequent analyses.

Table 1. Frequency of donor cells detected in BM, spleen, and PB of irradiated mice 3 hours after transplantation is positively correlated with the number of graft cells injected

<table>
<thead>
<tr>
<th>Cell dose</th>
<th>10^6</th>
<th>10^7</th>
<th>10^8</th>
<th>10^9</th>
<th>10^10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.05</td>
<td>3.30</td>
<td>24.54</td>
<td>0.599</td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.33</td>
<td>5.31</td>
<td>28.48</td>
<td>0.992</td>
</tr>
<tr>
<td>PB</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>1.17</td>
<td>1.06</td>
<td>26.06</td>
<td>0.996</td>
</tr>
</tbody>
</table>

PB indicates bone marrow; PB, peripheral blood.

*Numbers of PHK298* syngeneic low-density BM cells injected into single irradiated recipient mice.

*Numbers represent the percent of donor graft cells detected by flow cytometry in BM, spleen, and PB at 3 hours after transplantation. The lower limit of detection was <0.01%: n = 1 for each value.

*Correlation coefficient of linear regression analysis of cell dose and percent of donor cells detected in BM, spleen, and PB.
performed on donor Sca-1+ cells exhibiting light scatter properties characteristic of primitive HPCs (low side and forward scatter). Frequency of lineage-negative, CD43+, CD49e+, CD49d+, and CD62L+ BM-homed donor Sca-1+ cells increased in irradiated mice from 1 to 24 hours after transplantation, while these frequencies mostly fluctuated in irradiated mice (Figure 3A-E). CD11a and CD44 were present on 95% to 100% of donor Sca-1+ cells in the graft and all tissues examined and did not differ in irradiated and nonirradiated mice (n = 3-11, data not shown). At 20 to 24 hours, a slightly higher frequency of Sca-1+ cells in nonirradiated mice expressed CD43, CD49e, and CD49d and were CD3-CD45R/B220- (Figure 3), matching a phenotype enriched for long-term engrafting cells. Interestingly, while approximately 45% to 55% of donor Sca-1+ graft cells and those remaining in PB at 1 hour expressed CD62L, the majority of Sca-1+ cells recovered from BM at 1 hour lacked CD62L expression (Figure 3E).

Recovery and serial transplantation of donor cells in ENG cell transplants

To examine the trafficking of primitive HPCs more directly, grafts composed of $4 \times 10^4$ to $3 \times 10^5$ sorted cells enriched for stem cell phenotype (Sca-1- lin CD49c+ or Sca-1- lin CD62L+) were tracked at 20 hours in irradiated and nonirradiated 1ST mice. Figure 4 shows representative dot plots of typical analyses of donor cells found in BM and spleen tissues and the range of frequencies of donor cells detected in all experiments in BM, spleen, and PB. Similar to increased recovery of graft cells in BM of nonirradiated recipients when LDBM grafts were transplanted, recovery of purified ENG phenotypes was higher in nonirradiated mice, while recovery in spleen and PB was similar in the 2 settings (Figure 5).

We next defined the in vivo trafficking patterns of HSCs in irradiated or nonirradiated mice by examining the long-term repopulating potential of BM-homed donor cells 20 hours after injection of ENG cells. To this end, BM-homed donor cells were isolated from irradiated or nonirradiated 1ST recipients, and 50 to 200 of these cells transplanted competitively into 1ST-irradiated recipients. No significant differences in chimera were detected in 1ST recipients of BM-homed donor cells from irradiated or nonirradiated 1ST mice (Figure 6A) up to 6 months after transplantation. However, when $2.5 \times 10^6$ LDBM cells from 1ST recipients were transplanted into 2LD mice, donor-derived chimera was significantly greater in recipients of BM-homed cells from nonirradiated 1ST recipients (Figure 6B), suggesting more efficient homing or better survival of primitive HPCs in nonmyeloablative marrow. BM-homed cells from both irradiated and nonirradiated 1ST mice were equally effective in providing multilineage engraftment in both 1ST and 2LD recipients and did not apparently differ in their relative contribution to either myeloid or lymphoid lineages (data not shown).
Figure 3. Frequency of lineage-negative, CD43+CD49e+CD49d−, and CD62L+ cells among donor Sca-1+ cells in BM and PB at 1, 3, 6, and 20 to 24 hours following transplantation in irradiated or nonirradiated recipients. Irradiated or nonirradiated mice received 10^7 to 10^8 LD50 cells, killed at the indicated time points, and donor Sca-1+ cells in BM and PB analyzed for lineage expression (A), expression of CD43 (B), CD49e (C), CD49d (D), and CD62L (E) by flow cytometry as described in "Materials and Methods." PB samples were only analyzed at 1 hour because of infrequency of donor cells at later time points. Data are expressed as the mean ± SEM percent of donor Sca-1+ cells that have light scatter properties characteristic of primitive hematopoietic cells and that express each adhesion molecule. Horizontal bars on each graph represent the mean percent of Sca-1+ cells in the original graft that express each adhesion molecule. For each time point, n = 6-11 for irradiated BM, n = 3-6 for nonirradiated BM, n = 3-7 for PB, and n = 4-7 for graft cells. *P < 0.05 when compared with earlier time points of same transplant group. **P < 0.05 when compared with 1-hour BM values.

Cell cycle status

To examine the activation of primitive HPCs following transplantation, donor Sca-1+ cells from BM and spleen were analyzed for cell cycle status by PI staining of sorted cells or by BrdU incorporation. Figure 7 shows representative BrdU and cell cycle analysis of BM-homed Sca-1+ and Sca-1+lin+ cells from irradiated and nonirradiated recipients 6 hours after injection of LDBM. More than 95% of donor Sca-1+ cells isolated from BM or spleen were in G0/G1 regardless of time of analysis or irradiation status, a frequency similar to that of Sca-1+ cells in the original graft (Figure 8A-B). No significant differences in G0/G1 status were noted between irradiated and nonirradiated mice at different times of analysis.

Less than 22% of BM- and spleen-homed Sca-1+ cells from irradiated or nonirradiated mice were found to contain BrdU when analyzed between 1 and 24 hours, although this frequency declined by 24 hours in irradiated mice (Figure 8C-D). The more primitive Sca-1+lin− cells were also analyzed for BrdU incorporation and found to contain less than 10% BrdU+ cells regardless of irradiation status (data not shown). Of interest is that in 6 of 9 experiments, donor Sca-1+ cells homing to BM of nonirradiated recipients contained between 2- and 10-fold more BrdU+ cells than BM-homed Sca-1+ cells in irradiated marrow (Figure 8D-C). A similar trend of increased cycling in nonirradiated marrow was noted for the more primitive Sca-1+lin− cells and the more mature Sca-1-negative cells (3 of 5 and 7 of 7 experiments, respectively; data not shown).

Figure 4. Flow cytometric analysis of donor cells in BM and spleen 24 hours after transplantation of purified Sca-1+lin− CD45+ cells into irradiated or nonirradiated C57BL/6 mice (CD45.2+) received 1-5 × 10^7 sorted Sca-1+lin− CD45+ cells of B6/Rij origin (CD45.1+). irradiated 20 hours later, and donor cells in harvested BM and spleen detected by flow cytometry as described in "Materials and Methods." The frequency of donor cells is given in the upper left quadrant of each dot plot and was calculated manually using event count from listmode files containing between 5 × 10^4 and 1 × 10^5 events. The range of frequencies of detected donor cells in all experiments is given in the table.

Table 1. Summary of results

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Frequency of Donor Cells</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>BM</td>
<td>0.002-0.031</td>
<td>0.003-0.008</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.016-0.081</td>
<td>0.004-0.057</td>
</tr>
<tr>
<td>PB</td>
<td>0.001-0.041</td>
<td>0.002-0.008</td>
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</table>

Figure 5. Percent recovery of donor cells in BM, spleen, and PB 20 hours after transplantation of purified Sca-1+lin− CD45+ cells into irradiated or nonirradiated C57BL/6 mice. Irradiated or nonirradiated mice received 4 × 10^7 to 5 × 10^8 sorted Sca-1+lin− CD45+ cells of B6/Rij origin (CD45.1+), killed 20 hours later, and percent recovery of donor cells in harvested BM, spleen, and PB was calculated as described in "Materials and Methods." Bars represent mean ± SEM recovery of cells in each tissue; n = 11 for irradiated, n = 4 for nonirradiated.

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Discussion

In this report, we define the trafficking patterns and adhesion molecule repertoire of classes of phenotypically defined primitive BM cells early after their transplantation into lethally irradiated or nonirradiated recipient mice. Higher recovery of transplanted cells and increased incidence of CD43+, CD49e+, CD49d+, and Lin− BM-homed Sca-1+ cells in nonirradiated mice relative to irradiated mice suggests more efficient homing or better survival of primitive HPCs in a nonirradiated environment. The primitive nature of these cells was further substantiated in serial transplantation experiments, where BM-homed cells in nonirradiated mice were found to be relatively enriched for long-term engrafting cells capable of sustaining long-term hematopoiesis for 2 generations. Our results are in agreement with those recently reported by Bubnic and Keating, who documented the homing of long-term repopulating cells to BM of nonirradiated mice by 24 hours after transplantation in a similar transplant model.

The increase in CD43, CD49e, and CD49d expression on BM-homed Sca-1+ cells in nonirradiated mice correlates with the enhanced engraftment potential of BM-homed cells in nonirradiated mice and supports the earlier studies and those of others, suggesting an importance of these molecules in homing or engraftment of primitive HPCs. Whether these molecules are involved in homing and/or anchorage of primitive HPCs to BM or in modulating a yet-to-be-identified parameter important in engraftment remains to be determined. Nevertheless, these data suggest that while homing in myeloblastoid recipients may represent a random process due to radiation damage of stromal and endothelial cells, homing in nonmyeloblastoid recipients may not only be more specific, but may also better portray natural trafficking patterns of HSCs in vivo. Recent studies in parabiotic mice support the notion that a small number of HSC naturally traverse between blood and marrow in normal mice, further suggesting the existence of established migratory pathways for HSC between blood and BM in nonablated hosts. Our data suggest that these pathways are at least partially disrupted after lethal irradiation, such that fewer HSC home to, or survive within, an irradiated BM microenvironment. The reported generalized up-regulation of adhesion molecule counterreceptors, such as VCAM, ICAM, and PECAM, after irradiation may contribute to nonspecific seeding of transplanted HSC to sites other than BM.

Preliminary studies in our laboratory suggest that shorted time intervals between radiation dosing and transplantation may have favorable outcomes for homing and possibly engraftment, supporting the notion that radiation-sensitive microenvironmental cues may be involved in the homing process and fate of transplanted cells. Whether

![Image](https://example.com/image1)

**Figure 2. Flow cytometric analysis of BrdU incorporation in donor Sca-1+ and Sca-1+ cells in irradiated and nonirradiated recipient BM, and cell cycle analysis of donor Sca-1+ cells in irradiated recipient BM 6 hours after transplantation.** S5 × 10^6 BM cells of B6.BrdU origin were transplanted into irradiated or nonirradiated C57Bl6 mice. Mice were killed 6 hours later, and BM cells were lysed and stained with anti-BrdU-PE, Sca-1-biotin, CD3-cyocrome, and N-APC, followed by streptavidin-PE. Cells were permeabilized and stained with anti-BrdU-PE as described in Materials and Methods. A) and B) represent typical analyses, where donor cells (5.5%) are gated from all nucleated cells and analyzed for Sca-1 and lineage expression (B). Donor Sca-1+ cells (77% of donor cells; solid region in B) were then analyzed for their BrdU incorporation in irradiated (D) or nonirradiated (F) mice, or cell cycle status by PI staining (typical cell cycle histograms shown in G). Donor Sca-1+ cells (21% of donor Sca-1+ cells; dotted region in B) were likewise analyzed for BrdU incorporation (E). Sca-1+ cell histograms contain between 75 and 85 events, whereas Sca-1+ cell histograms contain between 250 and 701 events. Frequencies of BrdU+ cells and cells in S/G2 are given on each histogram.
radiation-induced bystander effects (reviewed in Mothersill and Seymour) negatively impact the function of transplanted primitive HPC and account for some of our observed differences in homing and engraftment in irradiated and nonirradiated mice is unknown. Nevertheless, our finding that graft cells home more efficiently to nonirradiated BM may partially explain the recent successes of BM transplantation in this scenario. A Better understanding of homing mechanisms may open these areas to manipulation and the possible design of protocols aimed at enhancing trafficking of transplanted HSCs to BM, which may be especially beneficial to those patients undergoing minimally ablative BM transplantation.

This report examined the adhesion molecule repertoire of phenotypically defined Sca-1+ cells early after transplantation. Although analysis of adhesion molecule expression on Sca-1+ cells would have yielded more complete knowledge of the homing patterns of primitive HPCs, analysis of such rare donor cell populations in recipient mice is technically difficult. However, the finding that CD62L−Sca-1+ cells, known to be enriched for long-term engraftment potential, rapidly homed to BM within 1 hour while the more mature CD62L+ cells remained in PB suggests the rapid trafficking of primitive HPCs in both irradiated and nonirradiated hosts. The dynamic changes in adhesion molecule expression observed on BM-homed donor Sca-1+ cells between 1 and 24 hours after transplantation suggest some of the differences in homing and engraftment seen in long-term bone marrow transplantation.

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Research Report

Hematopoietic Reconstitution of Irradiated, Stem Cell-Injected Mice:
Early Dynamics of Restoration of the Cell Lineages
of the Spleen and Bone Marrow

SANDRA C. MILLER

ABSTRACT

Hematopoietic stem cells, numbering approximately 1/100,000 cells in mammalian bone marrow, are capable of complete hematopoietic and immune reconstitution upon injection into a myeloablated host. The present studies aimed to analyze the earliest events in reconstitution of lethally irradiated host murine bone marrow and spleen, after injecting purified Thy 1"Lin-Sea-1" stem cells. Thy-1"Lin-Sea-1" cells were isolated by fluorescence-activated cell sorting (FACS) from the bone marrow of 4-week-old C57BL/6J-Thy 1.1, 1.1,5.1 mice and injected into preirradiated, syngeneic hosts. These stem cells were also injected into congenic hosts, i.e., C57BL/6-1.1, 1.5.2), and confirmed the donor origin of hematopoietic cells in the reconstituted host mice. Hematologically stained smears of the spleen and bone marrow of stem cell-injected recipients were prepared at 11, 14, 17, 21, 24, and 28 days after stem cell injection, and nucleated erythroid cells, mature granulocytes, and their myeloid precursors, monocytoids, and large and small lymphocytes were recorded as a proportion of all nucleated cells in each organ at each time interval. The results indicated that in the earliest post-stem cell injection intervals, both organs were predominantly erythroid and myeloid. Only at the later intervals did both organs show high proportions of large lymphoid cells and their progeny, small lymphocytes. Thus, early (<1 month) dynamics of hematopoietic reconstitution after transplantation of purified hematopoietic stem cells, is cell lineage specific.

INTRODUCTION

The concept and definition of the pluripotent stem cell stands without controversy as a cell that (1) reconstitutes lethally irradiated hosts into which it is injected, (2) is capable of self-renewal, and (3) enables full reconstitution of all hematopoietic and immune cell lineages in the recipient. Such pluripotent stem cells in the bone marrow occur with a frequency of 0.5-1/106 bone marrow cells (<1-3). Successful separation of primitive stem cells by means of the phenotype Thy-1"Lin-Sea-1" has been achieved (<1). The aim of the present work was to follow the relative reappearance of several categories of hematopoietic and immune cell lineages in both the bone marrow and the spleen at selected, frequent intervals throughout the first month after lethal irradiation and injection of irradiated syngeneic mice with only 500 Thy-1"Lin-Sea-1" stem cells. This early, post-transplant time period represents an important, critical phase in which survival or death of the transplant animal is often determined. The additional advantage of studying the dynamics of return of the various hematopoietic and immune cell lineages in this model system derives from the fact that the transplant, consisting of only purified stem cells, is uncomplicated by overwhelming numbers of committed precursors and mature forms for the various hematopoietic lineages, all of which are mixed with very few stem cells in normal bone marrow grafts.

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Long-term bone marrow culture data are the most powerful predictor of peripheral blood progenitor cell mobilization in healthy donors

Background and Objectives. There is wide interindividual variation in progenitor cell mobilization. The present study was aimed to analyze steady state hematopoiesis in healthy donors and its influence on hematopoietic progenitor cell (HPC) mobilization.

Design and Methods. Bone marrow (BM) was aspirated from 72 healthy donors prior to administration of recombinant human granulocyte colony-stimulating factor (G-CSF). Analyses of CD34+ cells and semisolid cultures as well as long-term cultures were performed from BM or leukapheresis products.

Results. Male donors showed a higher number of BFU-E (p=0.007) and committed progenitors (p=0.05), a better stromal layer (p=0.02), and higher long-term bone marrow culture (LT-BMC) counts (p=0.05) when compared to those in female donors. When correlating the culture pattern of the BM with the data from the leukapheresis products, we observed that the number of the immature progenitors in BM correlated significantly with both the number of CD34+ cells and CFU-GM in the first leukapheresis. Univariate analysis revealed that the following variables had a beneficial impact on the number of CD34+ cells: male sex, body weight >73 Kg, G-CSF schedule and results of LT-BMC; although in the multivariate analysis only the number of CFU-GM obtained after LT-BMC showed a significant influence (p<0.001).

Interpretation and Conclusions. These results confirm the interindividual variation in HPC mobilization among healthy subjects, with LT-BMC counts being the most reliable predictor, expressing the behavior of the immature progenitors and their relationship with the microenvironment.

Key words: healthy donors, long-term bone marrow cultures, CD34+ cells, hematopoietic progenitor cells, mobilization.

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Peripheral blood progenitor stem cells (PBSC) obtained after granulocyte-colony stimulating factor (G-CSF) administration are being increasingly used for autologous and allogeneic transplantation. Allogeneic transplantation using peripheral blood instead of bone marrow stem cells is associated with faster neutrophil and platelet recovery, without a higher incidence of acute graft-versus-host disease (GvHD). These events are probably due to a higher number of hematopoietic progenitor cells (HPC). Glimm et al. have also reported that PBSC contain a higher number of short-term repopulating hematopoietic cells (STRC) that engraft sequentially in NOD/SCID-β2 microglobulin-null mice. However, several studies indicate substantial interindividual variation in HPC mobilization among healthy donors. Some donor characteristics such as age, sex and weight, as well as apheresis volume or G-CSF schedule have been identified as variables influencing CD34+ cell collection, but there are several studies showing that there are poor mobilizers even among young, healthy donors. As far as we know, the in vitro behavior of immature progenitor cells in healthy individuals has not been extensively analyzed by long-term bone marrow cultures (LT-BMC). Although cell cultures (especially LT-BMC) are not routinely performed in transplantation centers, the results of such assays are important for understanding the behavior of different types of progenitors and the mobilization pattern, which is more important from theoretical and intellectual points of view than it is of practical use.

The aim of the present study was to analyze healthy donors in order to ascertain whether there are individual characteristics influencing steady state hematopoiesis behavior. We also wanted to analyze whether hematopoietic parameters
could influence the final yield of leukapheresis products. For these reasons, the percentages of CD34+ cells, committed HPC and immature HPC (assessed by LT-BMC) were evaluated in bone marrow cells prior to mobilization and their influence on leukapheresis yields was assessed.

Design and Methods

Donors
Bone marrow cells and leukapheresis products from 72 healthy donors, considered for allogeneic hematopoietic stem cell transplantation were analyzed. The donors' characteristics are shown in Table 1. Their median age was 41 years (range 11 to 72) with a male/female ratio of 44/28. After informed consent had been obtained, a bone marrow aspiration was performed in all donors before starting recombinant human G-CSF. Donors received G-CSF at a dose of 5 μg/kg/day (19% of cases) or 5 μg/kg/12h (81% of cases) over at least four days, until the apheresis procedures had been completed according to the policy of our center; both protocols were used sequentially and each donor received the dose active at that moment. The collection of peripheral blood stem cells was started on the fifth day of G-CSF administration. The blood volume processed was two to three times the donor's total blood volume. Donors underwent 1 to 3 leukaphereses (median 1). Procedures were performed using a continuous flow cell separator (CS-3000 plus, Baxter Healthcare corp., Deerfield, USA or COBE Spectra, COBE Inc., Colorado, USA). All patients were included in this study protocol after signed consent had been obtained according to the ethics committee of our institution. Flow-cytometry studies and cell cultures were performed in all healthy donors.

Flow cytometry studies
Whole bone marrow or leukapheresis product samples were stained using a stain-and-then-lyse direct immunofluorescence technique for analysis of the proportion of CD34+ cells, as previously described. Data were acquired in two consecutive steps on a FACScalibur (Becton Dickinson Biosciences, San Jose, USA) flow cytometer using the CellQuest software (BDB). In the first step, a total of 20,000 events/tube were acquired, and in the second step, acquisition throughout an electronic live-gate drawn on CD34+ cells was performed. In this latter step, 3x10⁶ events were measured with information only obtained from those events fulfilling the live-gate criteria. The Paint-A-Gate PRO software program (BDB) was used for data analysis.

<table>
<thead>
<tr>
<th>Table 1. Donors' characteristics.</th>
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<tbody>
<tr>
<td>N</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>72</td>
</tr>
<tr>
<td>Body weight (kg)</td>
</tr>
<tr>
<td>73 (28-108)</td>
</tr>
<tr>
<td>Number of aphereses performed</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>n=52</td>
</tr>
<tr>
<td>n=16</td>
</tr>
<tr>
<td>n=4</td>
</tr>
<tr>
<td>Number of mononuclear cells in 1° apheresis x10⁶/kg</td>
</tr>
<tr>
<td>5.3 (0.3-10)</td>
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<tr>
<td>Total number of MNC in 1° apheresis x10⁴</td>
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<tr>
<td>3.9 (0.2-8.6)</td>
</tr>
<tr>
<td>Number of CD34+ cells in 1° apheresis x10⁶/kg</td>
</tr>
<tr>
<td>3.8 (0.3-11)</td>
</tr>
<tr>
<td>Total number of CD34+ cells in 1° apheresis x10⁶</td>
</tr>
<tr>
<td>28.4 (6.9-80.1)</td>
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</table>

Results expressed as median (range).

Cell cultures
Mononuclear cells from bone marrow and leukapheresis products were used for semisolid and long-term cultures using methods previously reported. For clonogenic assays, mononuclear cells (MNC) were separated by centrifugation on a Ficoll Hypaque gradient (Lymphoprep™, Norgaard Co., Oslo) (d=1.077 g/mL). Interface cells were washed and resuspended on IMDM supplemented with FCS. The cells for long-term cultures were obtained by gravity sedimentation using a solution of 0.1% methylcellulose. The cells remaining in suspension were washed with IMDM-FCS.

Clonogenic assays
These assays were carried out, according to previously described methodology, in order to evaluate the committed HPC (CFU-GM, BFU-E and CFU-Mix) from bone marrow. In the leukapheresis products only CFU-GM were analyzed. We considered as CFU-GM colonies those colonies with a translucent appearance that contained granulocytes or macrophages or both. In our laboratory, colonies are considered to be more than 40 cells in an aggregate, whereas smaller numbers (5<40 cells) are considered to be clusters. The BFU-E colonies displayed a burst configuration of three to eight closely arranged erythroid clusters (mature BFU-E) or of more than eight clusters (primitive BFU-E), both dependent on erythropoietin being added to the culture. CFU-Mix colonies usually contained an erythroid component as well as cells from two of the other myeloid lineages. CFU-Mix showed a focus of hemoglobinized red cells and, at the same time, areas of dispersed translucent cells similar to parts of the granulocyte-macrophage colonies.
Table 2. Semi-solid and LT-BMC from steady-state bone marrow (n=72).

<p>| | |</p>
<table>
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<tbody>
<tr>
<td>CFU-GM*</td>
<td>196±184</td>
</tr>
<tr>
<td>BFU-E*</td>
<td>85±13</td>
</tr>
<tr>
<td>CFU-Mix*</td>
<td>6±13</td>
</tr>
<tr>
<td>Total CFU-GM in LT-BMC*</td>
<td>1086±7345</td>
</tr>
<tr>
<td>Total cells in LT-BMC (&gt;10^9)*</td>
<td>6.3±2.3</td>
</tr>
<tr>
<td>% Stromal layer*</td>
<td>9.9±7.1</td>
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</table>

*Number of colonies/10^6 cells plated. **Total number of CFU-GM and MNC obtained during the whole period of LTBMSC/10^6 cells plated. Results expressed as mean±SD.

Table 3. Relationship between the final numbers of CFU-GM obtained from LT-BMC and committed progenitors in BM.

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<tr>
<td>CFU-GM from LT-BMC and total number of progenitors (CFU-GM, BFU-E, CFU-Mix) in BM</td>
<td>0.54</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CFU-GM from LT-BMC and total number of CFU-E in BM</td>
<td>0.48</td>
<td>&lt;0.001</td>
</tr>
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</table>

All the tests were performed in all patients (n=72).

Cultures were performed as previously described. Plating efficiency was defined as the number of colonies produced by 10^6 plated cells.

Long-term bone marrow cultures

LTBMSC were established in order to analyze the immature HPC in bone marrow and were carried out according to the method of Gartner and Kaplan with slight modifications. Briefly, 2x10^6 cells/ml were inoculated in tissue culture flasks in LTBMSC medium IMDM supplemented with 10% pre-selected fetal calf serum (PCS, Biowhittaker, Belgium), 10% horse serum (HS, PAA Laboratories, GmbH, Austria) and 5x10^-6 M hydrocortisone sodium succinate. The cultures were incubated in a humidified atmosphere with 5% CO2 in air at 33°C for eight weeks. At weekly intervals before re-feeding, the stromal layer formation was studied under an inverted microscope. The degree of confluence and the presence of adipocytes and cobblestone areas of hematopoiesis were assessed and four stromal layer subtypes were established: (i) with all cellular components; (ii) without adipocytes; (iii) without adipocytes or cobblestone areas; and (iv) without stromal layer formation. For re-feeding, half of the supernatant was removed and replaced with fresh LTBMSC medium. The non-adherent cells harvested were quantified and assayed for their CFU-GM content. After 8 weeks of culture the whole supernatant was removed and the adherent layer was detached by exposure to trypsin. Cells were recovered, washed, counted and assayed for their CFU-GM content.

Long-term cultures from leukapheresis products

These assays were performed in a two-step procedure: stromal layers were established with bone marrow from a volunteer donor. When at least 70% of the flask’s surface was covered by the layer, the flask was irradiated with 15 Gy in a Cobalt bomb (Theratron 780). A second inoculum to obtain hematopoiesis was constituted by leukapheresis mononuclear cells. CFU-GM progenitors were evaluated during 8 weeks, as for the one stage long-term culture.

Statistical analysis

Statistical computations were carried out with the SPSS 10.0 program. The following tests were used: Student’s T-test for unpaired data, Pearson’s or Spearman’s test for quantitative correlation and multivariate stepwise analyses.

Results

PBSC collection results

As shown in Table 1, only one apheresis procedure was necessary for the majority of donors (72%) and three aphereses were necessary in only four cases. The mean number of mononuclear cells and CD34+ cells obtained in the first leukapheresis was 5.3±1.9x10^6/kg and 4.5±2.3x10^6/kg, respectively.

Results from bone marrow samples

Results of semisolid and long-term cultures obtained from steady state donor bone marrow samples are shown in Table 2. The sum of all committed progenitors (CFU-GM+BFU-E+CFU-Mix) obtained from 10^6 plated cells was 285±265, with CFU-GM being the most frequent progenitors and CFU-Mix the least frequent. In LT-BMC the production of CFU-GM was extremely heterogeneous, as reflected by the wide range of the number of colonies (374 to 31601 colonies/10^6 plated cells).

The mean percentage of CD34+ cells obtained in bone marrow was 0.93±0.05. No significant correlation between these values and the number of bone marrow progenitors (either committed, analyzed by semisolid cultures, or immature, analyzed by LTBMSC) was obtained. By contrast, when the steady-state hematopoiesis was analyzed, there was a clear, positive correlation between the numbers of committed stem cells and the number of immature ones analyzed by LT-BMC (Table 3).

Certain donor characteristics were associated with differences found in the number of colonies obtained both in the semisolid cultures and the LT-BMC (Table 3).
Table 4. Influence of donor characteristics on the number of progenitor cells in bone marrow.

<table>
<thead>
<tr>
<th>Donor characteristics</th>
<th>CFU-GM (&gt;10^6 cells plated)</th>
<th>BFU-E (&gt;10^6 cells plated)</th>
<th>CFU-Mix (&gt;10^6 cells plated)</th>
<th>Total progenitors (&gt;10^6 cells plated)</th>
<th>% SL in LTBMCS (&gt;10^6 cells plated)</th>
<th>Components of SL (&gt;10^6 cells plated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>218±129</td>
<td>105±105</td>
<td>6±11</td>
<td>328±314</td>
<td>80±15</td>
<td>0.98±0.15</td>
</tr>
<tr>
<td>F</td>
<td>184±199</td>
<td>53±91*</td>
<td>6±16</td>
<td>219±136*</td>
<td>63±29*</td>
<td>0.82±0.39*</td>
</tr>
<tr>
<td>Body weight</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;73 kg</td>
<td>174±129</td>
<td>73±99</td>
<td>3±8*</td>
<td>268±173</td>
<td>73±20</td>
<td>0.94±0.23</td>
</tr>
<tr>
<td>&gt;73 kg</td>
<td>217±230</td>
<td>96±108</td>
<td>9±17</td>
<td>321±326</td>
<td>74±22</td>
<td>0.83±0.52</td>
</tr>
<tr>
<td>CD34+ in BM</td>
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<tr>
<td>&lt;0.93</td>
<td>15±96</td>
<td>64±32</td>
<td>10±72</td>
<td>225±128</td>
<td>69±19</td>
<td>0.86±0.36</td>
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<tr>
<td>&gt;0.93</td>
<td>184±199</td>
<td>104±187</td>
<td>9±16</td>
<td>206±130</td>
<td>67±20</td>
<td>0.79±0.43</td>
</tr>
</tbody>
</table>

All the tests were performed in all patients (n=72). Results are expressed as mean±SD per 10^6 cells plated for CFU-GM, CFU-E, CFU-Mix and total progenitors, and per 10^6 cells plated for %SL. *p<0.05; **p<0.01. % SL: percentage of surface covered by the stromal layer. Components of SL: 1= all components present in the stromal layer (normality).

4). For example, when a fixed number of cells was plated, compared to female donors, male donors had a higher number of BFU-E (p=0.007), higher number of total progenitors in semisolid cultures (p=0.05) and a better stromal layer formation (p=0.02) with more cellular components. Moreover, donors with a body weight above 73 kg produced more progenitors in culture, although differences only reached statistical significance for CFU-Mix (p=0.04). When the age of donors and percentage of CD34+ cells in bone marrow were analyzed, no statistical differences were found for any of the cut-off points used.

In the LTBMCS studies, we observed that male donors showed higher cell counts (both CFU-GM and mononuclear cells) for values analyzed each week, and they also showed greater stroma formation with more cellular components, which reflects a better microenvironment. These differences between LTBMCS from male and female donors were statistically significant (p<0.05).

Results from leukapherases

Table 5 shows the plating efficiency and absolute numbers of CFU-GM generated both in the first and in all subsequent leukapherases performed. A wide range of inter-individual variation was observed. The median number of CFU-GM in the first leukapheresis was 211 colonies /10^6 cells plated (range 9-1800). (Table 5)

Table 5. Results of cell cultures from leukapherases products.

<table>
<thead>
<tr>
<th></th>
<th>N. of CFU-GM/10^6 cells plated in the first leukapheresis</th>
<th>N. of CFU-GM/10^6 cells plated in all leukaphereses</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>211 (9-1800)</td>
<td>252 (38-2540)</td>
</tr>
</tbody>
</table>

Total number of colonies CFU-GM in the 1st leukapheresis (>10^6)

|                      | 270 (7-1450)                                             |

Total number of colonies CFU-GM in all leukaphereses (>10^6)

|                      | 360 (5-1450)                                             |

All the tests were performed in all patients (n=72). The results are expressed as the median (range).

Number of CD34+ cells (n=56; p<0.0001) (Figure 1), and, to a lesser extent, CFU-GM present in the first leukapheresis product (r=0.45; p=0.001) and in the final apheresis (r=0.40; p=0.006). By contrast, neither the committed bone marrow progenitor cells nor the percentage of CD34+ cells in bone marrow showed a significant relationship with the quality of the apheresis.

Factors influencing the PBPCSC collection

In order to ascertain their predictive value, we analyzed a series of clinical and culture assay variables. Univariate analysis revealed that the following variables had a beneficial impact on the yield of CD34+ cells or mononuclear cells (Table 6): male sex, body weight above 73 Kg, dose of 5 µg/kg/12h of G-CSF and a higher number of CFU-GM in LTBMCS. Furthermore, male sex was associated with a lower number of leukaphereses, with differences close to statistical significance (p=0.06). In order to ensure that the donor's body weight did not condition the differences in results, the parameter was independently analyzed and produced similar results with only one new variable, sex, showing an impact on
CFU-GM yields ($p=0.017$). When a multivariate analysis was performed in order to assess which variables could have an independent impact on CD34+ cell mobilization, only the total number of CFU-GM generated after LTBM showed a significant influence ($p<0.001$). This significance was maintained when the body donor’s weight was considered.

### Discussion

Long-term cultures are used to analyze primitive hematopoietic stem cells and their relationship with the bone marrow microenvironment, while clonogenic assays are used to analyze committed hematopoietic progenitor cells. Taken together, both types of assay offer a comprehensive measurement of marrow function.

Peripheral blood hematopoietic progenitor cells have been successfully employed in autologous and allogeneic transplantation and their use has been associated with a significantly faster hematologic recovery than that afforded by bone marrow progenitor cells. This effect is probably related to the fact that the leukapheresis product contains several fold more progenitor cells than does bone marrow but also because higher numbers of STRC are present in mobilized peripheral blood. However, it has frequently been reported that a wide interindividual variation exists in the characteristics of peripheral blood progenitor mobilizations among healthy donors. This is why continuous studies are carried out on steady-state hematopoiesis in healthy donors. In the present work we analyzed how different parameters of steady-state hematopoiesis, as well as other individual characteristics (such age or weight) or the G-CSF schedule, may influence final leukapheresis yields. Regarding the behavior of bone marrow progenitors, a great degree of variability in the

<table>
<thead>
<tr>
<th>Table 6. Factors influencing progenitor cell mobilization (univariate analysis).</th>
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<tr>
<td>Sex</td>
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<tr>
<td>Male</td>
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<td>Age</td>
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<tr>
<td>&lt;42</td>
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<tr>
<td>&gt;42</td>
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<tr>
<td>Body weight</td>
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<tr>
<td>&lt;73</td>
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<tr>
<td>&gt;73</td>
</tr>
<tr>
<td>G-CSF</td>
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<tr>
<td>5</td>
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<tr>
<td>5/12h</td>
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<tr>
<td>%CD34&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>&lt;0.3</td>
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<td>&gt;0.3</td>
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</table>

All the tests were performed in all patients (n=72). Results expressed as mean±SD; *p<0.05. N.V.: not valuable. Total n of CFU-GM in LTBM/ total number of CFU-GM obtained during the whole period of LTBM=10×10⁶ cells plated.
plating efficiency and number of immature progenitors in bone marrow samples from healthy donors was observed, showing that there is considerably interindividual variability in hematopoiesis. Interestingly, differences were particularly striking between sexes with males showing a higher production of all types of progenitors and, moreover, a better stroma confluence, and hence a better microenvironment. As far as we know, this feature (the influence of donor sex on the number of all hematopoietic progenitors and stroma formation) has not been previously reported. However, a higher proportion of erythropoietic precursors in men has been related to the stimulatory effect of androgens.#

The second goal of the present study was to analyze whether steady-state hematopoiesis could influence the yields of leukapheresis. We found that the only parameter which correlated with the final number of progenitor cells obtained in leukaphereses was the number of CFU-GM during LTC-MBC. It is well known that these progenitors reflect the number of immature hematopoietic progenitors in bone marrow, which, theoretically, will be mobilized into the peripheral blood after G-CSF administration. Our results confirm, using a multivariate approach, those published recently by Carlo-Stella et al. who showed that steady state marrow LTC-IC had a clear relationship with the CD34+ and CFU-GM cell yields in leukapheresis after G-CSF mobilization.

In previous studies it was observed that some donor characteristics influenced peripheral blood progenitor cell mobilization. From Data from the Spanish Registry showed that age (above vs below 38 years) and G-CSF schedule (once vs twice a day) are the most powerful predictors of CD34+ cell mobilization. However, poor mobilizers have been observed in all age groups. In the present analysis the clinical factors that influenced the peripheral blood mobilization were: age, male sex, G-CSF schedule, higher body weight and a higher percentage of CD34+ cells in the bone marrow. However, these influences on mobilization were only observed using univariate analysis. The influence of age has been observed by other authors, in fact, older donors show a poorer apheresis yield. It should be noted that among our group only 5 donors were older than 60 years. When a multivariate analysis was performed, the only variable that retained independent predictive value was the number of immature progenitors, as detected by LT-BMC cultures. Previous analyses in the autologous transplantation setting have shown the relevance of immature progenitors on peripheral blood cell recovery. In the present study these results were not confirmed in the allogeneic setting. This could be due to the fact that a second apheresis was performed when not enough cells were obtained with the first apheresis and this could have disguised the real impact of the immature progenitor cells.

Taken together, our results confirm the interindividual variation in progenitor cell mobilization among healthy subjects. Some individual donor characteristics (sex, G-CSF schedule) can influence cell yields after mobilization. However, the only independent parameter that influences mobilization is the production of CFU-GM in LTMC which reflects the behavior of the immature progenitors and their relationship with the microenvironment. Although this kind of analysis is of no clinical value because of the long period that is required for their results, our analysis shows that there are poor mobilizers, even among young donors, because of an inefficiency in mobilizing hematopoiesis. These endogenous differences in hematopoiesis and their possible causes are of great interest for further analysis and point to new ways to overcome such problems in healthy donors.

NLH, CP and MCC were responsible for the conception of the study and interpretation of results. NLH and CP performed the statistical analysis and wrote the manuscript. All authors critically revised the paper and give the final approval for its submission. The order in which the names of the authors appear is based on their contribution to the study. JPSM and MCC, as heads of department and laboratory, are cited last. The authors declare that they have no potential conflict of interest.

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CD34\(^+\) cell subsets and long-term culture colony-forming cells evaluated on both autologous and normal bone marrow stroma predict long-term hematopoietic engraftment in patients undergoing autologous peripheral blood stem cell transplantation

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Objective. The aim of this study was to evaluate which CD34\(^+\) cell subset contained in leukapheresis products could be regarded as the most predictive of long-term hematopoietic recovery after autologous peripheral blood stem cell transplantation (auto-PBSCT).

Materials and Methods. Based on data from 34 patients with hematologic malignancies, doses of CD34\(^+\) cells and CD34\(^+\) cell subsets, defined by the expression of HLA-DR, CD38, CD117 (c-kit), CD123 (a subunit of IL-3/IL-3R), CD133 (AC133), and CD90 (Thy-1) antigens, were correlated with the number of short-term (i.e., colony-forming cells [CFC]) and long-term culture CFC (LTC-CFC) generated at week 5 of culture and with the kinetics of hematopoietic engraftment following auto-PBSCT. The capacity of autologous stroma (AS), normal human bone marrow stroma, and M2-10B4 marine cell line to sustain CD34\(^+\) cell growth was comparatively evaluated in the LTC assay.

Results. Our data demonstrated that some of the most primitive progenitor subsets (CD34\(^+\)CD117\(^-\)HLA-DR\(^-\), and CD34\(^+\)CD38\(^-\)HLA-DR\(^-\)) showed the strongest correlation with LTC-CFC numbers generated within the AS, whereas no significant correlation was noted using normal bone marrow stroma. Multivariate analysis showed that the only CD34 cell subset independently associated with long-term (3 to 6 months) platelet engraftment after auto-bone marrow transplantation was the CD34\(^+\)CD117\(^-\)HLA-DR\(^-\) phenotype; long-term erythroid engraftment was correlated with CD34\(^+\)CD38\(^-\)HLA-DR\(^-\) cell content. The latter further influenced platelet engraftment in the first 3 months after auto-PBSCT. The most predictive parameters for neutrophil engraftment were CD34\(^+\)CD38\(^-\)HLA-DR\(^-\) cell subtype and the total LTC-CFC quantity infused.

Conclusions. These data further support the hypothesis that the type of stromal feeders influences the frequency of LTC-CFC, possibly because they differ in their ability to interact with distinct subsets of hematopoietic stem cells. Furthermore, as the use of AS in LTC assay can mimic in vitro the human bone marrow microenvironment, it can be speculated that this culture system could be a useful means to study the kinetics of recovery of bone marrow stroma following chemotherapy and PBSCT. From these results, it can be concluded that some CD34\(^+\) cell subsets appear to be more reliable predictors of long-term hematopoietic recovery rates than total CD34\(^+\) cell quantity. © 2001 International Society for Experimental Hematology.
Long-term culture (LTC) of bone marrow (BM) cells have been used as an in vitro model of hematopoiesis to study the interactions among early progenitors, stroma, and regulating factors [1–4]. Different stromal layers have been used in LTC assays, and recent data have shown that the type of stromal feeder used affects the frequency and maintenance of long-term culture colony-forming cells (LTC-CFC) [5]. Transformed murine and human engineered feeder cell lines, such as M2-10B4 [6], MS5 [7], S17 [8], and FBMD-1 [9] secreting supplementary growth factors, are widely used in the LTC assay, even if their ability to support the growth of hematopoietic stem cells (HSC) was found to differ from cell line to cell line [8–10]. Some authors showed that human primary BM cultures [11,12] are able to generate stromal layers that are more efficient in supporting HSC growth than the mouse-derived feeders, even if a regular and reliable provision of normal BM may present practical difficulties to many laboratories. Alternative methods are represented by the use of either cryopreserved normal BM [13] or preformed stroma and autologous stroma (AS) [14]. The use of stromal-dependent LTC has demonstrated the importance of the adherent layer as a reservoir of the most primitive stem cells and that a direct contact between stromal and hematopoietic cells is critical for stem cell growth and differentiation [15–17].

Although CD34+ cells expressing little or no CD38 and lacking HLA-DR antigen define a primitive subpopulation of progenitors in fetal liver, leukapheresis products (LP) [18,19], cord blood, and BM [20,21], at the present time it is not known which progenitor cell subsets are optimally predictive of short- and long-term engraftment after autologous peripheral blood stem cell transplantation (autoPBSC) [22–28]. Most published reports have focused on the analysis of early post-transplant multilineage recovery [29–31], while the influence of CD34+ subsets and LTC-CFC cells on long-term engraftment has not yet been clearly defined [32]. Interestingly, absent stromal layer development was found to be associated with poor hematologic recovery after bone marrow transplantation (BMT) [33].

The aim of this study was to evaluate which CD34+ cell subsets contained in LP could be regarded as the most predictive of long-term hematopoietic recovery after auto-PBSC. To reach this goal, we correlated CD34+ cell subsets with the numbers of both CFC and LTC-CFC generated within allogeneic normal BM stroma (NBMS), M2-10B4 cell line, and AS layers after 5-week culture of selected CD34+ cells obtained from LP of 34 patients with hematologic malignancies undergoing auto-PBSC. In addition, the different CD34+ cell subsets defined by highly sensitive flow cytometry were tentatively correlated with the number of LTC-CFC obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after 5 weeks of culture. Finally, the total CD34+ cell quantity and doses of CD34+ cell subsets and LTC-CFC infused were correlated with the kinetics of hematopoietic recovery over a 6-month follow-up period after auto-PBSC.

Materials and methods

Patients and PBSC collection

PBSC were obtained from 34 patients (19 men and 15 women; median age 44 years, range 25 to 58) in remission: 24 with non-Hodgkin's lymphoma (NHL), 5 Hodgkin disease (HD), and 5 multiple myelomas (MM).

Patients were treated according to the following chemotherapy regimen: MACOP-B followed by sequential high-dose chemotherapy (Milan protocol) for NHL, ABVD schedule for HD, and VAD regimen for MM.

CD34+ cells were mobilized with chemotherapy as follows: 26 patients with high-dose cyclophosphamide (7 g/m²) plus granulocyte colony-stimulating factor (G-CSF, lenograstim or filgrastim, 5 μg/kg body weight/day), and 8 patients with intermediate-dose cyclophosphamide (4 g/m²) plus G-CSF (5 μg/kg body weight/day). Mean percentage of harvested CD34+ cells in LP was 3.23 ± 2.28 (range 1.1–12.6). The conditioning regimens adopted in this study for PBSC were mitoxantrone 60 mg/m² plus melphalan 180 mg/m² for NHL and MM, and BEAM regimen for HD. G-CSF administration started 5 days after PBSC and continued until absolute neutrophil count was >1,000/μL for 3 consecutive days. Mean interval between the end of preconditioning therapy and the mobilization regimen was 36 days (range 30–65). BM samples were collected at least 4 weeks after the last chemotherapy regimen to minimize the effect of cytotoxic drugs to the patient's BM microenvironment.

Mean number of CD34+ cells infused was 6.54 × 10⁹/kg body weight (range 2.65–11.0 × 10⁹/kg body weight). Three patients were transfused with a CD34+ cell dose <5 × 10⁹/kg.

Short-term engraftment was evaluated according to standard criteria (neutrophil count >500 μL and platelet count >20,000/μL on at least three consecutive analyses). Long-term recovery after auto-PBSC was assessed by evaluating the hemoglobin (Hb) level, white blood cell count, neutrophil count, and platelet count at different time intervals (1, 3, and 6 months after PBSC). Secondary graft failure was considered to have occurred if, on 3 consecutive days after full engraftment was documented, granulocytes decreased to <50/μL, and/or platelets to <20,000/μL. Incomplete recovery was considered for platelet count <50,000/μL and/or neutrophil count <1,000/μL.

PBSC were collected on a Baxter CS3000 cell separator (Baxter, Milan, Italy) using a continuous collection procedure until 2.5× the patient's blood volume had been processed [34]. The target value was 4 × 10⁸ CD34+ cells/kg. LP were cryopreserved with 10% dimethylsulfoxide and autologous plasma and stored in liquid nitrogen.

Controls. Normal BM was obtained from proximal epiphysis of eight subjects (mean age 49 years; male/female ratio 1:1) who underwent major orthopedic surgical intervention.

Purification of CD34+ cells from LP

Enriched CD34+ cell fractions were obtained by immunomagnetic separation (Mini-MACS CD34 isolation kit, Miltenyi, Bergisch Gladbach, Germany), as previously described. A mean of 7 × 10⁸ PBSC were separated on LymphoFlow-H 1.077 g/cm³ gradient (Cedarside Laboratories Limited, Hornby, Ontario, Canada), then incubated with an hapten-conjugated CD34 monoclonal antibodies (mAbs) (QBEND-10), followed by incubation with magnetic microbeads conjugated to an anti-hapten antibody. The positive frac-
tion and unseparated cells were stained with CD34 epitope class III-reactive mAb (HPCA2-FITC). CD34+ cell purity was measured by flow cytometry. Recovery, purity, and enrichment factor were calculated according to standard protocols [35].

Flow cytometric analysis of CD34+ cell subsets from LP
Immunomagnetically selected CD34+ cells were analyzed with a Facsscan flow cytometer (Becton-Dickinson, San Jose, CA, USA). The instrument was calibrated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (Per-CP) beads provided by Becton-Dickinson. Data were analyzed with appropriate negative (isotypic) controls using CellQuest and Paint-a-Gate research software [36,37]. At least 20,000 cells were analyzed for each sample.

The CD34+ cell population was identified using a combined approach based on multicolor analysis and evaluation of light-scattering properties of the cells [38]. Flow cytometric data were expressed as percentage of positivity (using standard marker approach). Expression of the various antigens by CD34+ cells was evaluated by direct immunofluorescence using a multiparametric approach based on the use of the following combination of mAbs: CD34 (HPCA-2)-FITC/CD38 (HB-7)-PE/HLA-DR (clone L243)/Per-CP; CD34-FITC/IL-3/Receptor (clone 7G3)-PE/HLA-DR/Per-CP; CD34 (HPCA-2)-FITC/AC133-PE; CD34-FITC/CD117 (c-Kit)/R clone: 95C3)-PE/HLA-DR/Per-CP; CD34-FITC/CD90-PE (Thy-1, SE10)/HLA-DR/Per-CP. CD34, CD38, and HLA-DR mAbs were provided by Becton-Dickinson, CD90 and CD123 mAbs were purchased from PharMingen (San Diego, CA, USA), and AC133 was purchased from Miltenyi.

Determination of doses for the various CD34+ cell subsets were calculated by calculating the percentage of total CD34+ cells that expressed the appropriate marker of interest and multiplying that percentage by the CD34/kg values that had been previously determined.

Short-term colony assays from BM and LP samples
BM mononuclear cells (MNC) were isolated by centrifugation on Lymphoprep-H gradient, washed in phosphate-buffered saline, and plated in triplicate at a density of 1 x 10^6 cells in 35-mm Petri dishes (Bn, Milan, Italy) in 1.1 mL of a methylcellulose semisolid medium (Megacult H4343; Stem Cell Technologies Inc., Vancouver, Canada). Petri dishes were incubated at 37°C in a fully humidified atmosphere with 5% CO2 and scored at day 14 under inverted microscope (Wilovet-Wil, Wetzlar, Germany) for the presence of CFU (colony-forming unit granulocyte-macrophage [CFU-GM], burst-forming unit erythroid [BFU-E], multilineage colony-forming unit [CFU-GEMM]). Short-term colony assays also were performed seeding 0.5 x 10^5 cells/mL obtained from LP, as previously described.

BM stromal feeders
Patient microenvironment from 41 patients (including the 34 cases investigated by multivariate analysis in this study) affected by NHL (n = 28), HL (n = 7), and MM (n = 6) were investigated by CFU-fibroblast (CFU-F) assay [39]. Briefly, 1 x 10^9/mL BM-MNCs were resuspended in LTC medium containing Iscove modified Dulbecco’s medium (IMDM; Euroclone Ltd., Paington, UK) with presel ected 12.5% fetal bovine serum, 12.5% horse serum (both sera from Stem Cell Technologies, Vancouver, Canada), 1% L-glutamine (Euroclone), and 1% penicillin-streptomycin (Euroclone), and plated on 35-mm collagenated bio coated Petri dishes (Becton-Dickinson). The medium was changed every week, and cultures were incubated at 37°C in a humified atmosphere supplemented with 5% CO2. Fibroblast aggregates with >30 cells were scored as CFU-F. All determinations were performed in duplicate and expressed as mean values. The same method was used for detecting and scoring spontaneous endothelial colonies (CFU-Endo), according to a previously described technique [40].

Human primary feeder layers were obtained according to a modified version of the Gartner and Kaplan method [1]. BM-MNC cells were seeded at a density of 1 x 10^7 in T12.5-cm^2 plastic tissue culture flasks (Falcon) in LTC medium supplemented with fresh 5 x 10^-6 mol/L hydrocortisone sodium succinate (Sigma, St. Louis, MO, USA). After 20 to 30 days of culture, the confluence of the stromal layers was checked under inverted microscope and assessed by a semi-quantitative scoring system. The degree of confluence from the various culture systems was expressed as percentage of adherent cells covering the bottom of the flask (range 0-100%). Established autologous and normal stromal feeders, at least confluent for 70%, were irradiated at 16 Gy at the first passage of the culture. The stromal layers from 7 of 41 patients examined did not achieve the minimum level (70%) of confluence and, therefore, were not used for estimation of LTC-CFC. Thus, LTC-CFC was evaluated only in 34 patients.

The murine stromal nontransfected cell line M2-10B4 was maintained in RPMI 1640 medium (Euroclone) supplemented with 10% fetal bovine serum (Euroclone). At confluence, M2-10B4 cells were irradiated at 60 Gy. LTC-CFC assay on the M2-10B4 cell line was investigated in a smaller group of 12 patients (8 NHL, 2 HD, 2 MM). For this reason, statistical analysis between LTC-CFC numbers generated on M2-10B4 cells and CD34+ cell subsets and hematopoietic engraftment after PBSCT was performed in this study.

When a confluent stromal layer was achieved, selected CD34+ cells were seeded in T12.5 cm^2 plastic tissue culture flasks (Falcon) on the different irradiated stromal layers at a density of 3 x 10^6 cells in LTC-medium at 37°C and a humidified atmosphere with 5% CO2. Stromal-contact cultures were fed weekly by changing half of the medium. Nonadherent cells recovered from the supernatant were assayed in short-term methylcellulose assay for the presence of committed progenitors. At week 5 of culture, both nonadherent and adherent cells, after treatment with trypsin and after stromal cell depletion, were likewise washed and separately assayed for LTC-CFC content, as described earlier.

The immuno phenotypic characteristics of stromal cells were assessed using immunohistochemical technique with a wide range of mAbs [41].

Statistical analysis
Using the Pearson test, we first compared the percentage of positivity for the various phenotypic markers expressed by mobilized CD34+ cells with the number of week 5 LTC-CFC generated within the different stromal layers. In a further analysis, the Pearson test was used to examine the relationship among the incidence of LTC-CFC generated on AS, doses of total CD34+ cells and CD34+ cell subsets infused, and hematologic parameters (HB value, platelet count, white blood cell count) used to predict long-term engraftment after auto-PBSCT. The incidence of CFC present in PBSCT preparations was correlated with doses of CD34+ cells, CD34+ cell subsets, and clinical parameters at different time intervals after PBSCT. In all cases, the confidence interval was deter-
minded to identify which parameters showed the strongest and most constant correlations. A confidence range with \( p < 0.05 \) was considered significant. To assess whether a certain cell dose could predict for long-term hematopoietic recovery, we calculated the mean doses of the various CD34+ cell subsets transplanted in patients with normal and delayed long-term hematopoietic engraftment. Multiple regression analysis was used to determine which of the CD34+ subsets or LTC-CFC numbers are the best independent predictors for the long-term engraftment.

The number of CFC, CFU-F, CFU-En, and percentage of stromal layer confluence in controls and patients groups were compared using nonparametric statistics (Wilcoxon test).

**Results**

**Immunophenotypic profile of selected CD34+ cells**

Flow cytometric analysis of the selected CD34+ cell population showed a mean purity of 81.3% (range 60–99.7%) and a mean recovery of 60% (range 33–81%). Multicolor analysis of purified CD34+ progenitor cells obtained from PBSC is given in Table 1. In brief, the lowest mean percentage of CD34-expressing cells was found in the CD34+CD38- HLA-DR+ cell fraction (0.12%), as well as in the CD34+CD38+ HLA-DR+ (0.18%), CD34+CD123+ HLA-DR+ (0.32%), CD34+CD90+ HLA-DR+ (0.49%), and CD34+CD38+ HLA-DR+ (1.1%) cell subsets. The highest values were found among the following CD34+ cell subsets: CD34+CD38- HLA-DR+ (98.5%), CD34+CD123+ HLA-DR+ (92.2%), CD34+CD117+ HLA-DR+ (77.3%), and CD34+AC133+ (75.9%).

**Short-term colony assays of BM and LP samples**

No significant difference was found between the clonogenic potential of BM cells from patients in remission and controls. Cell morphology and colony size were comparable in the two groups. Mean values of the clonogenic potential of hematopoietic progenitors obtained from LP are given in Table 2.

**Clonogenic mesenchymal progenitors and stromal confluence assays**

With regard to the microenvironment, patients showed a significantly lower CFU-F number than controls (\( p = 0.003 \)) (Table 2). The incidence of CFU-En found to grow spontaneously in LTC assay was higher in BM samples obtained from NHL and MM patients compared with controls (1.5 vs 0.45; \( p = 0.01 \)) and other patient categories, as previously described by our group [41]. We also evaluated the capacity of the autologous and normal BM stromal cells to form a confluent monolayer usable as primary feeder layer in the LTC assay (Table 2). Healthy control stromal layers always reached the confluence (100%), whereas BM specimens from the patient group displayed a variable confluence degree (range 70–100%). However, in 7 (17%) of 41 samples analyzed (4 NHL, 2 HD, 1 MM), the stromal layer confluence capacity was particularly impaired (mean 35%, range 10% to 55%). Cells from these cases were not used as feeder layers in the LTC-CFC assay and, therefore, were excluded from this study.

**LTC-CFC assay**

The number of committed progenitors generated from week 1 to week 5 of culture was assessed, and the results were subdivided according to the type of stromal layer used in the LTC assay. As shown in Figure 1A, the mean numbers of CFU-GM/10^5 CD34+ cells derived from AS (1416.2 at week 1 of culture to 251.5 at week 5) were found to be higher than those on NBMS (906.8 at week 1 of culture to 165 at week 5) and M2-10B4 cell line (732.2 at week 1 to 61.9 at week 5); however, these differences were found to be not significant, except at week 1 comparing CFU-GM on AS vs those on M2-10B4 (\( p = 0.01 \)). The frequency of BFU-E (Fig. 1B) from the M2-10B4 cell line was lower (42 at week 1 to 0 at week 5) than that on AS (179.2 at week 1 [\( p = 0.01 \)] to 3.2 at week 5) and NBMS (121.8 at week 1 to 0.64 at week 5). No significant differences in the incidence of CFU-GEMM among AS (21 at week 1 to 0 at week 5),

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<th>Table 1. Immunophenotypic profile of selected CD34+ cells from apheresis products of patients with hematologic malignancies</th>
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<tr>
<td>Mean ± SD</td>
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<td>Range</td>
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<tr>
<td>CD34+/90+/DR+</td>
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<td>Mean ± SD</td>
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<tr>
<td>Range</td>
</tr>
</tbody>
</table>

Data are given as percentage of CD34+ cells coexpressing the various phenotypic markers.
Table 2. Bone marrow and peripheral blood clonogenic assays in patients and controls

<table>
<thead>
<tr>
<th>Culture assay</th>
<th>BM patients Mean ± SD (range)</th>
<th>BM Controls Mean ± SD (range)</th>
<th>PBSC Patients Mean ± SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM</td>
<td>41 ± 18.2 (3.5–70)</td>
<td>52.7 ± 14.5 (25.3–72.5)</td>
<td>104 ± 87.8 (0–372)</td>
</tr>
<tr>
<td>BFU-E</td>
<td>36.9 ± 17.1 (12–66)</td>
<td>37.8 ± 18.3 (10–66)</td>
<td>67.4 ± 77 (0–295)</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>2.5 ± 2.4 (0–10)</td>
<td>2.6 ± 1.7 (0–5)</td>
<td>4.02 ± 4 (0–15)</td>
</tr>
<tr>
<td>CFU-F</td>
<td>9.1 ± 6.7 (2–24)</td>
<td>16.2 ± 5.06 (5–24)</td>
<td></td>
</tr>
<tr>
<td>CFU-En</td>
<td>1.5 ± 2 (0–8)</td>
<td>0.45 ± 0.93 (0–2)</td>
<td></td>
</tr>
<tr>
<td>Percent confluence</td>
<td>83.5 ± 25.7 (70–100)</td>
<td>100 ± 10 (80–100)</td>
<td></td>
</tr>
</tbody>
</table>

CFU-GM = colony-forming unit granulocyte-macrophage per 10^5 LP derived cells; BFU-E = burst-forming unit erythroid per 10^5 LP derived cells; CFU-GEMM = colony-forming unit granulocyte, erythroid, monocyte, and megakaryocyte per 10^5 LP derived cells; CFU-F = colony-forming unit fibroblast per 10^5 BMMNC; CFU-En = colony-forming unit endothelial colony per 10^5 BMMNC.

NBMS (16 at week 1 to 0.4 at week 5), and M2-10B4 (5 at week 1 to 0 at week 5) were found (Fig. 1C). Figure 1D shows the variation of total CFC number during the 5 weeks of culture of the LTC assay.

With regard to the total number of LTC-CFC after 5 weeks of culture, the highest value was seen on AS compared with both NBMS and M2-10B4, but these differences were not significantly different.

At week 5 of culture, we considered separately the number of LTC-CFC generated from nonadherent and adherent fractions. The mean values obtained from the adherent fraction were significantly lower than the values from the nonadherent fraction. The highest number of LTC-CFC from the adherent fraction was seen on AS, whereas lower values were documented in LTC-CFC on NBMS and, above all, on the M2-10B4 cell line (p values are given in Table 3).

Data collected from a smaller series of 12 patients showed a good reproducibility of our LTC-IC culture system. In fact, if the same CD34 sample was seeded onto three identically derived stromal support layers, the 5-week CFC activity on the three identically derived stromal layers was similar (p = NS).

Furthermore, purified CD34+ cell preparations obtained from different positive selection procedures were grown in triplicate on several AS preformed layers. Results were found to be comparable in all culture systems (CV < 7%; p = NS).

In three experiments, purified CD34+ cells from healthy subjects were grown onto AS and data were compared with

![Diagram](image_url)
allogeneic NBMS and M2-10 B4 cell line. LTC-CFC number was found to be higher on AS than on allogeneic NBMS and cell line (data not shown).

Correlation between CD34+ cell subsets, CFC from LP, and LTC-CFC from different stromal feeders
A strong correlation between the total number of CD34+ cells and of CFU-GM contained in the LP (p = 0.0001) was found. The incidence of CFU-GM from LP was correlated with the CD34+AC133+ cell subset (p = 0.03) and inversely with CD38+DR- (p = 0.048) (Table 4). No strict associations between CFC and other CD34 cell subsets were noted in our study.

With regard to LTC-CFC in vitro assay, the strongest and most constant correlations were observed between CD34+CD117+HLA-DR- (p = 0.016) and CD34+CD38+ HLA-DR+ (p = 0.02) progenitor cell subsets and LTC-CFC number, generated within the AS cell culture. No significant correlations were found between CD34+ cell subsets and LTC-CFC evaluated on NBMS.

With regard to the correlations between the clonogenic potential of CD34+ progenitors obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after week 5 of culture of the LTC assays and the different CD34+ cell subsets, we found that the CD34+CD38+DR- (p = 0.006) and the CD34+CD117+HLA-DR- (p = 0.016) cell subtypes showed a strong correlation with the adherent fraction generated within AS. No correlation were found with the non adherent fraction (Table 4).

Correlation between doses of CD34+ cells, CD34+ cell subsets, CFU-GM colonies, LTC-CFC infused, and kinetics of engraftment after auto-PBSCT
We correlated doses of CD34+ cells and CD34+ cell subsets infused with the incidence of LP-derived CFU-GM and LTC-CFC, and the kinetics of engraftment in the late posttransplant period (3 to 6 months after PBSCT). These data were compared with the following hematologic parameters: Hb level, and neutrophil, leukocyte, and platelet counts at 1, 3, and 6 months after auto-PBSCT.

With regard to clinical data, short-term engraftment occurred in all patients examined in this study (range 9–12 days after PBSCT). For long-term engraftment after PBSCT, 30 of the 34 patients achieved a neutrophil count >1,000/μL, and 29 achieved a platelet count >50,000/μL over a 6-month follow-up period. Within this patient group, two patients with NHL occasionally required platelet and red blood cell transfusions 2 to 6 months after PBSCT. Loss of platelet engraftment occurred in the same 4 patients who experienced a transitory loss of neutrophil engraftment. All five patients who presented a late loss of neutrophil and/or platelet engraftment received a number of CD34+ cells (6.21 × 10^6/kg) comparable to that infused into patients who achieved good long-term hematopoietic engraftment (6.87 × 10^6/kg CD34+ cells) (Table 5).

No significant correlation was found between the total CD34+ cell quantity and clinical parameters.

A positive correlation between the number of transplanted LTC-CFC tested on AS and platelet count 1 month after auto-PBSCT was noted (p = 0.048) (data not shown). Higher correlations were found between LTC-CFC tested on AS and platelet values at 3 to 6 months post-transplantation (p = 0.004). No significant correlation was found between doses of LTC-CFC on NBMS and hematologic parameters (Table 4).

With regard to CD34+ cell subset analysis, complete and durable platelet engraftment was correlated with CD34+CD117+DR- (p = 0.04) and CD34+AC133- (p = 0.027) cell subsets (Table 5). CD34+CD38+DR- cells also showed a direct correlation with both leukocyte (p = 0.025) and neutrophil count (p = 0.023) 3 to 6 months after PBSCT; CD34+AC133- cell doses were correlated with long-term (3 to 6 months) leukocyte (p = 0.03) and neutrophil (p = 0.02) engraftment; and CD34+CD123+DR- correlated only with neutrophil count (p = 0.038).

Table 5 shows that the numbers of CD34+CD117+DR-, CD34+CD38+DR-, and CD34+CD123+DR- cells/kg infused were significantly lower in the group of patients (n = 5) who had delayed or long-lasting loss of engraftment from month 3 to 6 after PBSCT. In contrast, all patients who had received higher doses of these cell subtypes experienced complete and durable platelet and neutrophil engraftment after PBSCT.

Multivariate analysis showed that the only CD34 cell subset independently associated with long-term (3 to 6 month) platelet engraftment after auto-BMT was the CD34+CD117+HLA-DR+ phenotype (b = 0.63, p = 0.039). A significant association between the CD34+CD38+HLA-DR+ cell content and platelet engraftment also was found, but this relation reached a significant level only within 3 months after auto-PBSCT (b = 0.49, p = 0.011). In contrast, the only parameter predictive for long-term erythrocyte engraftment was the CD34+CD38+HLA-DR+ cell content (b = 0.45, p = 0.023). In the first 3 months after auto-PBSCT, both CD34+CD38+HLA-DR- cell number (b = 0.77, p < 0.001) and the total LTC-CFC quantity infused (b = 0.51, p = 0.011) were associated with neutrophil engraftment. In contrast, no correlation was found between CD34+ cell quantity and long-term hematopoietic engraftment.

### Table 3. Adherent and nonadherent output of CFC at week 5 of LTC

<table>
<thead>
<tr>
<th>Strona type</th>
<th>Nonadherent fraction</th>
<th>Adherent fraction</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS (p = 0.002)</td>
<td>179.6</td>
<td>71.8</td>
<td>251.4</td>
</tr>
<tr>
<td>NBMS (p = 0.003)</td>
<td>129.2</td>
<td>35.8</td>
<td>165</td>
</tr>
<tr>
<td>M210B4 (p = 0.001)</td>
<td>49.4</td>
<td>7.36</td>
<td>61.9</td>
</tr>
</tbody>
</table>

*p Values in parentheses are relative to the comparison between the nonadherent and adherent fractions.

AS = autologous stroma; NBMS = normal bone marrow stroma.
Table 4. Correlation between number of CFU-GM, LTC-CFC, CD34+ cells, and CD34+ cell subsets in PBSC preparations from 34 patients with hematologic malignancies in relation to long-term engraftment

<table>
<thead>
<tr>
<th></th>
<th>Hematologic parameter 3–6 months after PBSCCT</th>
<th>Hb (g/dL)</th>
<th>WBC</th>
<th>Neut</th>
<th>Plt</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+</td>
<td>(r = 0.83)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD117+/HLA-DR+</td>
<td>(r = 0.44)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38+/HLA-DR+</td>
<td>(r = 0.54)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/AC133+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC-CFC* (week 5)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+</td>
<td>(r = 0.89)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD117+/HLA-DR+</td>
<td>(p = 0.016)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38+/HLA-DR+</td>
<td>(p = 0.048)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL) WBC Neut Plt</td>
<td>NS NS NS NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherent fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC-CFC* (week 5)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+</td>
<td>(r = 0.89)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD117+/HLA-DR+</td>
<td>(p = 0.02)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38+/HLA-DR+</td>
<td>(p = 0.006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonadherent fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTC-CFC* (week 5)</td>
<td>NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+</td>
<td>(r = 0.89)</td>
<td></td>
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</tr>
<tr>
<td>CD34+/CD117+/HLA-DR+</td>
<td>(p = 0.016)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38+/HLA-DR+</td>
<td>(p = 0.006)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb (g/dL) WBC Neut Plt</td>
<td>NS NS NS NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*LT-CFC evaluated on autologous stroma.

Hb = hemoglobin; NBMS = normal bone marrow stroma; nd = not done; Neut = neutrophil count; Plt = platelet count; WBC = white blood cell count.

Discussion

In this article, we assessed the predictive value of the total quantity of CD34+ cells and of the various CD34+ cell subsets obtained from LP for the long-term hematopoietic recovery after auto-PBSCCT in patients with hematologic malignancies in remission phase. To obtain this goal, we also tested the ability of selected CD34+ progenitors obtained from LP to produce progeny over long time spans by LTC assay on different stromal cell feeder layers. Because it has been postulated that BM microenvironment could play a role in HSC engraftment and considering that our LTC system was based on the use of both human primary autologous and normal BM stroma, we also examined the clonogenic potential of BM mesenchymal cells in the pretransplant period. Our data showed that cells from patients with hematologic malignancies could have a lower CFU-F incidence than that of normal BM, and occasionally a slower and reduced stromal layer formation capacity, compared with that of the control group, thus confirming previous reports [42]. However, these alterations do not necessarily lead to altered architecture of BM microenvironment or defective functional support for in vitro hematopoiesis, as further supported by our data from LTC assay on AS that exhibited a normal supportive capacity for stem cells. Previous studies documented that either chemotherapy or autologous and allogeneic stem cell transplantation could damage the BM microenvironment [43–46], and these alterations were found to be correlated with a very poor hematopoietic recovery after BMT [33]. However, based on our results, to obtain an efficient stromal layer from these patients, we would recommend collecting BM aspirates as far as possible from the time of administration of the cytotoxic drugs. This could

Table 5. Correlation between doses of CD34+ cells, CD34+ cell subsets, and long-term hematopoietic engraftment after PBSCCT

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 5) who had delayed long-term engraftment</th>
<th>Patients (n = 29) who had normal long-term engraftment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+ cells</td>
<td>Cell number transplanted/kg ((\times 10^6))</td>
<td>Cell number transplanted/kg ((\times 10^6))</td>
</tr>
<tr>
<td>Hb</td>
<td>WBC Neut Plt Mean Range p Value Mean Range</td>
<td></td>
</tr>
<tr>
<td>CD34+</td>
<td>NS NS NS NS 621 265–1100 0.006 687 410–1004</td>
<td></td>
</tr>
<tr>
<td>CD34+/CD117+/HLA-DR+</td>
<td>NS NS r = 0.54 3.4 0.3–8 0.006 27 7–134</td>
<td></td>
</tr>
<tr>
<td>CD34+/CD38+/HLA-DR+</td>
<td>NS NS r = 0.61 p = 0.04 2.9 0.6–9 0.049 9 4–23</td>
<td></td>
</tr>
<tr>
<td>CD34+/AC133+</td>
<td>NS NS r = 0.67 r = 0.61 r = 0.69 119.8 81–159 0.049 252 33–318</td>
<td></td>
</tr>
<tr>
<td>CD34+/CD123+/HLA-DR+</td>
<td>NS NS r = 0.54 p = 0.02 p = 0.027 8 5–18 0.042 13 4–37</td>
<td></td>
</tr>
</tbody>
</table>

Data are subdivided according to whether or not patients achieved good hematologic recovery 3–6 months after autologous transplant.
The two groups of patients with normal or delayed long-term engraftment were compared using Wilcoxon’s test.

AS = autologous stroma; NBMS = normal bone marrow stroma.
minimize the short-lasting negative effect of chemotherapy on the BM microenvironment.

With regard to the capacity of different stromal cell layers (AS, NBMS, and M2-10B4) to sustain the proliferation of selected CD34+ progenitors in the LTC-CFC assay, we observed a higher number of CFC generated within the AS and NBMS than those on M2-10B4 line, thus suggesting a specific release of BM human stroma-derived cytokines capable of inducing the differentiation and proliferation of progenitor cells. These data confirm previous findings indicating that the type of stromal feeder influences the frequency and maintenance of LTC-IC in different pathologies [5] and may suggest that certain cell types do not provide an ideal environment for stem cell growth [5,8,47].

Comparison between the expression of the various immunophenotypic markers (HLA-DR, CD38, CD117 [c-kit/R], CD123 (α subunit of IL-3/R), AC133, and CD90) on CD34+ cells and the in vitro incidence of LTC-CFC from LP showed that some of the more primitive CD34+ cell subtypes, such as CD34+CD117-DR- and CD34+738 HLA-DR-phenotypes) positively correlated with week 5 LTC-CFC numbers generated within the AS. No correlation was found between CD34+ cell subsets and week 5 LTC-CFC numbers from NBMS. These results further support the hypothesis that distinct stromal cell subtypes could in vitro interact selectively with different subsets of stem/progenitor cells, and that the various stromal cell feeders used in this study have a different ability to support in vitro stem cell growth, thus confirming previous results [48,49].

In this study, we also studied the differences in the clonogenic potential of selected CD34+ progenitor cells obtained from the adherent and nonadherent (i.e., supernatant) fractions collected after week 5 of culture of the various LTC assays. Our data confirm that some CD34+ subpopulations, such as CD34+CD38-HLA-DR- and CD34+CD117-HLA-DR- cells, have a preference to proliferate in close contact to human stroma layers and particularly to AS.

Based on our data and the fact that the homing process by HSC is rather specific, the use of AS in the LTC assay can be proposed in some selected cases to better investigate the in vivo interactions between HPC and BM stroma in patients undergoing BMT or PB SCT and for assessment of the kinetics of recovery of patient BM microenvironment after chemotherapy and/or PB SCT [50]. In contrast, NBMS had a prevalent capacity to select in vitro more committed CD34+ progenitor cells cultured in LTC assay. Our culture system also stressed the importance of assessing the progenitor content of the adherent layer, in line with two recent studies [51,52] that investigated the interaction between BM stroma and HSC by an expanding family of proteins, referred to as connexins. Our results could be explained by the occurrence of allogeneic mismatch between the selected CD34+ cells and the type of stromal cells used in LTC assay. Sugiru et al. [53] recently provided further evidence that formation of cobblestone colonies under major histocompatibility complex (MHC)-mismatched stromal cells significantly decreased compared with MHC-matched stromal cells. This was related to MHC class I molecule mismatch [53].

With regard to clinical data, until now no clear correlations between the incidence of transplanted LTC-IC, doses of CD34+ cell subsets infused, and long-term hematopoietic recovery after PB SCT were found [20,22,28,29], even if it is expected that LTC-IC would contribute more to later than early reconstitution following transplant [54]. Several authors found a positive correlation between the presence of certain CD34+ cell subpopulations and the incidence of LTC-IC in apheresis products [55], which is in accordance with our own data showing that complete and durable platelet engraftment after auto-PB SCT was found to be influenced by CD34+CD117-DR- cell content, whereas the only CD34+ cell subset independently associated with long-term neutrophil and erythrocyte recovery was the CD34+CD38- HLA-DR- phenotype. These results suggest that stem cell subpopulations defined by negativity for CD117 and HLA-DR and positivity for CD38 antigen appeared to be more reliable predictors of long-term hematopoietic recovery rates than total CD34+ cell dose infused, thus supporting the validity of our approach in this context. These CD34+ cell subsets were able to identify patients who experienced delayed engraftment 3 to 6 months after PB SCT. Interestingly, the same two CD34+ cell subsets showed the strongest correlation with LTC-CFC grown on AS, thus confirming the biologic role played by these progenitor cell subsets. Multivariate analysis also showed that in the first 3 months after auto-PB SCT, better neutrophil recovery was correlated with the number of transplanted LTC-CFC generated on AS. These in vitro data make a testable prediction that using NBMS or AS cells in LTC assay would improve the in vivo analyses of stem cell recovery after PB SCT. However, the patients examined in this study were mobilized with chemotherapy plus G-CSF. As a result, these conclusions may not apply to PB SCT mobilized with G-CSF alone.

At this point, it should be said that our functional and phenotypic analyses were performed on selected CD34+ cells. From a theoretical point of view, this could represent a bias of the study; however, some considerations must be made. First, it should be kept in mind that using both Ficoll and Percoll density gradients to establish culture assays causes a considerable loss of CD34+ cells [56,57]. Second, multicolor flow cytometric analysis of LP-derived CD34+ cell subsets is far from standardized, especially for samples having low percentages of CD34+ cells. Based on these data, we thought it noteworthy to estimate the presence of the various CD34+ cell subsets on purified CD34+ cells, to minimize these methodologic problems. Selective loss of progenitor subsets following CD34+ cell enrichment procedures has been documented in a few reports [58–60], raising the possibility that our analysis cannot predict the composition of the various progenitor cell subpopulations present in the whole LP.
In conclusion, these data further support the hypothesis that the type of stromal feeders influences the frequency of LTC-CFC, and that the use of human stromal layers could be more suitable for an in vitro study that more strictly analyzes the in vivo engraftment process following autologous PBSC. Furthermore, the CD34+ cell subsets defined by CD117, CD38, and HLA-DR appear to be more reliable predictors of long-term hematopoietic recovery rates than total CD34+ cell quantity. These data may help to predict the repopulation capacity of PBSC, especially when relatively low numbers of CD34+ cells/kg (<3 x 10^6/kg) are reinjected.

Acknowledgments
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The Elusive Peripheral Blood Hemopoietic Stem Cell

Donna E. Hogge, Heather J. Sutherland, Peter M. Lansdorp, Gordon L. Phillips, and Connie J. Eaves

Long-term culture initiating cells (LTC-IC) are primitive hematopoietic progenitors that give rise to clonogenic cells when provided with a supportive feeder layer of mesenchymal cells. These LTC-IC possess many of the characteristics expected of marrow-repopulating "stem cells" including high proliferative and multilineage-differentiative capacity and resistance to 4-hydroperoxycyclophosphamide (4-HC) killing. In addition, stem cells are known to persist and may proliferate in murine LTC, and human marrow grown in LTC has been successfully used as hematopoietic support for myeloablative therapy. LTC-IC, as well as clonogenic precursors, circulate in normal peripheral blood, and the concentration of both progenitor types can be increased by cytotoxic chemotherapy and/or growth factors. When mobilized peripheral blood cells are used as hematopoietic support for high-dose chemotherapy, engraftment has often been more rapid than that achieved with autologous marrow. Thus, primitive hematopoietic cells circulate in human blood, which can enable hematopoietic reconstitution following aggressive therapy for malignant disease.

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CHARACTERIZATION OF PRIMITIVE HUMAN HEMATOPOIETIC CELLS IN LONG-TERM CULTURE

The ongoing production of mature blood cells is the end result of the activity of primitive precursor cells termed hematopoietic stem cells. Stem cells are characterized both by their ability to generate more mature, differentiated progeny and by their capacity to self-replicate or self-renew. They are a population distinct from the progenitors that give rise to colonies of mature blood cells when placed in semisolid media with a source of hematopoietic growth factors. These latter cells are committed to differentiate down one or more lineages, and have limited proliferative capacity and little or no ability for self-renewal. In animals the "stem cell" function of a particular cell or population is assessed by its ability to reconstitute multilineage hemopoiesis in lethally irradiated recipients. Since in vivo assays are impractical in man, in vitro systems have been developed to allow the characterization and quantitation of primitive hematopoietic precursors. One such system termed the long-term culture (LTC) assay has been used by our group to study early events in human hemopoiesis.

When unseparated bone marrow cells are placed in culture at high concentration with appropriate medium but in the absence of added growth factors, an adherent layer of mesenchymal stromal cells (largely fibroblasts, endothelial cells, and fat cells) forms with which primitive hematopoietic progenitors become associated. These progenitors proliferate and differentiate over several months in culture, releasing their clonogenic (or colony-forming) and mature cell progeny (largely granulocytes and macrophages) into the culture medium. The ongoing production of these colony-forming units (CFU) and mature cells is the result of the differentiation and proliferation of primitive hematopoietic cells termed long-term culture initiating cells (LTC-IC). The presence of these LTC-IC can be detected and their number counted by assaying for the presence of CFU in cultures where hematopoietic cells are maintained on a feeder layer of bone marrow stromal cells for a minimum of 5 weeks. Beyond this time point, any colony-forming cells initially present in the culture should have disappeared through differentiation or death, and those detected will be the result of differentiation by LTC-IC.

In the absence of added growth factors, the maintenance of hematopoietic activity in LTC is dependent on the presence of the stromal feeder layer. When the hematopoietic population to be assayed is depleted of endogenous stromal cells (as is the case for fluorescence-activated cell sorter (FACS)-purified bone marrow cells) or does not normally contain such stromal cells (which is true for peripheral blood cells), a preestablished feeder must be provided. The total clonogenic content of the cultures after 5 weeks has a linear relationship with the number of hematopoietic cells from a given sample initially plated onto the

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feeder. The number of these CFU thus provides an indirect quantitation of LTC-IC.

Direct quantitation of LTC-IC and an assessment of their individual proliferative and differentiative potential can be obtained by placing limiting numbers of hemopoietic cells on feeders in microtiter wells. After 5 weeks of culture, the contents of each well are assayed for CFU. From such limiting dilution assays the frequency of LTC-IC in normal marrow has been determined to be one in 20,000 nucleated cells. The frequency in normal peripheral blood is approximately three LTC-IC/mL, or about 100-fold lower than the frequency in marrow as compared with other nucleated cells. In marrow the ratio of CFU to LTC-IC is normally about 8:1, whereas in the blood it is approximately 80:1 (Table 1). Individual LTC-IC produce an average of four clonogenic cells each regardless of whether they are isolated from marrow or blood. (Table 1). However, some LTC-IC from either source produce as many as 30 CFU. Although the majority of such CFU produced are granulocyte-macrophage progenitors (CFU-GM), many LTC-IC from marrow or blood produce CFU of more than one lineage, usually CFU-GM in combination with erythroid (burst-forming units) or mixed-lineage progenitors (CFU-GEMM).

The cell surface phenotype of LTC-IC has been used to partially FACS purify them from human marrow and blood. LTC-IC show low forward and side light scatter properties, and like clonogenic cells, are strongly CD34+. However, they differ from most CFU from bone marrow in that they are largely negative for HLA-DR or CD71 (transferrin receptor), and show weak retention of Rh-123. These characteristics are very similar in LTC-IC isolated from peripheral blood or marrow. However, CFU from blood differ from marrow CFU and are more similar to LTC-IC

from both sources in being HLA-DR and Rh-123-positive (Fig 1).

Bone marrow treated with 4-hydroperoxycyclophosphamide (4-HC) to remove malignant cells before autologous transplantation is depleted of CFU, but retains its marrow-repopulating (stem cell) capability. LTC-IC from marrow or blood are also 4-HC resistant. Although marrow CFU are 4-HC sensitive, their counterparts in peripheral blood survive treatment with the drug in a fashion similar to LTC-IC (Fig 2).

Thus, LTC-IC are primitive hemopoietic cells from which clonogenic cells are derived. They can be detected in both normal marrow and peripheral blood, although at much lower concentrations from the latter source. Many of the characteristics of LTC-IC suggest that they may be, if not identical to repopulating stem cells, at least closely related. These features include their immunophenotype, 4-HC resistance, multilineage potentiality, and the high proliferative capacity that some of them exhibit. In addition, repopulating stem cells are maintained in LTC initiated with mouse bone marrow, and human marrow

| Table 1. Quantitation and Differentiation of LTC-IC and Clonogenic Cells in Normal Peripheral Blood and Bone Marrow |
|-----------------|-----------------|-----------------|
| Cell Type       | Blood (per mL)  | Marrow (per mL) |
| CFU             | 231 ± 20        | 95 ± 14        |
| LTC-IC          | 2.9 ± 0.5       | 12 ± 3         |
| CFU per LTC-IC  | 3.7 ± 1.2       | 4.3 ± 0.4      |

NOTE: Results are means ± SEM.

| Table 2. Relative Numbers of Different Types of Clonogenic Cells Produced by LTC-IC From Normal Blood and Marrow After 5 Weeks in LTC |
|-----------------|-----------------|-----------------|
|                | BFU-E           | BFU-GM          |
| Blood           | 11 ± 2          | 89 ± 2          |
| Marrow          | 8 ± 2           | 91 ± 2          |

NOTE: Results are means ± SEM expressed as % of the total.

**Fig 1.** HLA-DR expression and Rh-123 retention of CFU and LTC-IC from normal bone marrow [3] and peripheral blood [9]. Means ± SEM of six and three experiments for marrow and blood, respectively.
maintained in LTC has been used to support high-dose chemo/radiotherapy for patients with leukemia.\textsuperscript{14} The existence of LTC-IC in the peripheral circulation as well as marrow suggests that blood might also be used to support myeloablative therapy in man.

HEMOPHOIETIC RECONSTITUTION WITH PERIPHERAL BLOOD PROGENITOR CELLS

It has been known for many years that clonogenic hematopoietic progenitors are present in the peripheral blood of animals and human beings.\textsuperscript{15-17} Thus, even before LTC-IC had been demonstrated, a number of investigators had suggested that blood cells may have hematopoietic repopulating ability. Successful marrow reconstitution with peripheral blood progenitor cells (PBPC) in mice, monkeys, and dogs preceded the first studies in man.\textsuperscript{15,16,18}

In chronic myelogenous leukemia (CML), the concentration of CFU in the blood is enormously enriched over that in normal blood. McCarthy and Goldman\textsuperscript{19} used cells harvested from the blood of patients with CML in chronic phase to support high-dose chemo/radiotherapy for the same patients when their disease had advanced to the accelerated stage. Chronic-phase hematopoiesis was reestablished readily in most such patients. Although the short duration of the remissions achieved from the accelerated phase has led to this approach being largely abandoned for treatment of CML, these studies did demonstrate the successful use of PBPC for autologous transplantation.

Unfortunately, several initial attempts to use normal peripheral blood for transplantation were less successful, with two patients experiencing graft failure following PBPC transplants for aplastic anemia.\textsuperscript{20,21} Nevertheless, there was strong rationale for pursuing attempts to optimize peripheral blood harvests for hematopoietic reconstitution. First, a significant number of patients with advanced malignancy who would otherwise be candidates for myeloablative therapy cannot undergo marrow harvests, due to marrow involvement with their malignancy or previous irradiation to marrow harvest sites. Second, the ability to avoid general anesthesia and the trauma of the surgical procedure associated with marrow harvests is important in some patients. In addition, apheresis technology has improved sufficiently in the last decade to allow efficient processing of the large volumes of blood required to obtain adequate numbers of PBPC for transplantation. Finally, even in the early series some patients transplanted with autologous blood showed extremely rapid and sustained count recovery. The possibility that PBPC transplants would allow more rapid engraftment than marrow cells has been and continues to be one of the strongest motivations for the use of such cells to support high-dose chemo/radiotherapy for patients with malignant disease.

However, there were concerns about the use of peripheral blood that retarded the widespread adoption of PBPC transplants initially. The development of graft failure in some of the early patients treated with PBPC harvested during steady-state hematopoiesis was almost certainly due to the infusion of inadequate cell numbers. How-
ever, although it was clear that larger numbers of cells were needed to ensure engraftment when blood rather than marrow was used as a source of hemopoietic support, the optimum dose of cells or even the type of cells needed was unknown. In response to this uncertainty, investigators were forced to collect large numbers of cells requiring multiple apheresis procedures per patient. The collection and cryopreservation of these products were cumbersome and time consuming as compared with that for a single marrow harvest. Nevertheless, for certain groups of patients the rationale for PBPC transplants was compelling, and experience with the use of this modality has gradually accrued over the last decade. For example, in 1988 Kessinger et al. reported on a series of patients receiving PBPC transplants to support therapy for advanced, refractory Hodgkin's disease. In their group of 10 patients, median count recoveries on days 22, 27, and 23 for neutrophils numbering more than $0.5 \times 10^9/L$, hemoglobin levels greater than 10 g/dL, and platelets numbering more than $20 \times 10^9/L$, respectively, were comparable to what was typically reported for autologous marrow transplants. Although several patients showed delayed platelet recovery, the overall recovery times were very encouraging in this group of heavily pretreated patients.

**Mobilization of PBPC for Autologous Transplantation**

It had been known for some time that CFU numbers increase in the peripheral blood as the white blood cell (WBC) count increases following the nadir induced by cytotoxic chemotherapy. Juttner et al. were among the first to report a series of patients who received autotransplants of PBPC collected during the rebound that occurs following chemotherapy. In a series of eight patients with acute nonlymphoblastic leukemia who had PBPC collected during their recovery from remission-induction chemotherapy and subsequently used to support a high-dose conditioning regimen, count recoveries occurred at a median of 11, 12, and 14 days for neutrophils numbering more than $0.5 \times 10^9/L$, platelets numbering more than $50 \times 10^9/L$, and lymphocytes numbering more than $0.5 \times 10^9/L$. These times were significantly shorter than the investigators' previous experience with autologous or allogeneic marrow grafts for the same disease. This and the experience of others suggested the potential usefulness of PBPC in decreasing the morbidity and mortality associated with myeloablative therapy.

Clonogenic cells can also be mobilized into the blood of animals and man with the use of hemopoietic growth factors. Socinski et al. showed that patients receiving granulocyte-macrophage colony-stimulating factor (GM-CSF) had a greater than 10-fold increase in the concentration of CFU-GM and a fourfold increase in BFU-E concentrations in the blood. In the same study, they demonstrated the synergistic effect on CFU concentrations of combining GM-CSF with chemotherapy. One hundred-fold increases in CFU-GM concentrations were achieved in patients receiving both modalities.

Gianni et al. harvested PBPC from patients receiving GM-CSF following high-dose cyclophosphamide therapy. These cells were used to supplement autologous bone marrow for support of high-dose therapy in patients with advanced-stage but previously untreated Hodgkin's disease. Count recovery was very rapid in patients receiving PBPC as compared with that in patients receiving marrow alone, with medians of 9 and 11 days for recoveries of neutrophils numbering more than $0.5 \times 10^9/L$ and platelets numbering more than $50 \times 10^9/L$, respectively. These results were very encouraging, but it was not clear whether they could be repeated in different patient groups, particularly in those who had been heavily treated with previous courses of chemotherapy. It was also uncertain whether autologous marrow was necessary for long-term engraftment or whether PBPC alone could provide both short- and long-term hemopoietic support.

Over the last several years, we have treated 18 patients with PBPC alone to support high-dose therapy for advanced, malignant disease. PBPC were harvested in six patients during steady-state hemopoiesis, in six patients during count recovery following 7-g/m² cyclophosphamide therapy, and in six patients following cyclophosphamide plus GM-CSF therapy by continuous intravenous infusion. All of these patients had received one or more courses of chemotherapy with or without radiotherapy before PBPC harvests, and some had marrow involvement with their malignancy. We evaluated the kinetics of mobilization of both CFU and LTC-IC following cyclophosphamide
treatment. Rebound of CFU concentration to a mean of sevenfold above the normal range began 15 to 25 days after cyclophosphamide. The speed of rebound was several days faster in patients receiving GM-CSF in addition to cyclophosphamide (Fig 2). LTC-IC rebound followed kinetics similar to those of CFU, although it tended to occur several days earlier and peaked at a mean of fourfold above normal levels (Figs 3 and 4).

Leukapheresis was performed using a CS 3000 (Baxter Fenwal Division, Deerfield, IL) or Spectra (Cobe Laboratories, Lakewood, CO) blood cell separator. Nine liters of blood were processed over 2 to 3 hours, allowing collections of 5 to 10 \( \times 10^9 \) mononuclear cells. Following cyclophosphamide treatment, collections were begun when the total WBC count had increased after the nadir to more than \( 1.0 \times 10^9 /L \). A mean of seven collections were performed for patients harvested during steady-state hemopoiesis or for those receiving cyclophosphamide with or without GM-CSF (Table 3). Although the total number of mononuclear cells harvested was greater in patients harvested during steady-state hemopoiesis, the number of CFU was significantly increased in patients receiving cyclophosphamide mobilization. The mean number of CFU per kilogram was increased more than 13-fold in patients receiving GM-CSF plus cyclophosphamide. LTC-IC numbers harvested were also increased in patients receiving cyclophosphamide with or without GM-CSF, but the magnitude was less than that seen for CFU.

The PBPC collected from all 18 patients were used to support high-dose chemotherapy for the same patients. The speed of count recovery for neutrophils and platelets was compared with the dose of CFU and LTC-IC infused with the autograft. As shown in Table 4, most patients achieved recovery of both cell types, but in some cases recovery of neutrophils and/or platelets was delayed. In patients receiving cyclophosphamide, the speed of total WBC rebound during mobilization (days to WBC > \( 1.0 \times 10^9 /L \)) predicted the speed of engraftment following reinfusion of PBPC. In general, patients with the lowest CFU and LTC-IC doses had the longest recovery times. In six patients achieving count recovery by day 15, the dose of CFU infused (18 to 360 \( \times 10^9 /L \))
kg) was higher than in the four patients where WBC and/or platelet recovery was delayed past 25 days (1.0 to 12 \times 10^5/kg). Although the mean LTC-IC number infused was highest for patients with rapid engraftment, the range of LTC-IC dose overlapped with that seen for patients with delayed recovery. Thus, LTC-IC numbers do not appear to improve on CFU dosage in predicting time to count recovery posttransplant. From these data, it also appears that a dose of 20 \times 10^4 CFU/kg predicts rapid engraftment of neutrophils post-PBPC transplants. This dosage is less reliable in predicting platelet recovery, perhaps reflecting the fact that we assessed CFU-GM and BFU-E but not megakaryocyte precursors in our peripheral blood harvests. Juttner et al have also observed a relationship between CFU numbers infused and posttransplant recovery times.

The assessment of CFU numbers takes approximately 2 weeks, making its use to assess the quality of collections impractical in many clinical situations. We and others have assessed the possibility of using CD34+ cell numbers to indirectly assess the numbers of progenitors in peripheral blood collections. Because CD34+ cells are present at such low numbers as to be difficult to detect in normal blood, this method cannot be used for steady-state collections. However, when mobilization techniques such as cyclophosphamide with or without GM-CSF are used, the proportion of CD34+ cells increases dramatically, allowing their reliable enumeration (Table 2). In such samples, the number of CFU shows strong correlation with CD34+ cell numbers (r = .95). However, LTC-IC numbers show only a very weak correlation (r = .28). Thus, in mobilized PBPC collections, CD34+ cell numbers can allow rapid assessment of the CFU content of the autograft.

Growth factors other than GM-CSF are being used to mobilize PBPC. For example, Sheridan et al have used G-CSF–mobilized PBPC to supplement bone marrow autografts for patients with advanced malignancy. In such patients, there was a dramatic increase in the speed of platelet recovery and a concomitant decrease in platelet transfusion requirements as compared with that for patients receiving autologous marrow followed by G-CSF administration. This improvement in platelet recovery appears to be significantly better than what has been observed historically with GM-CSF–mobilized cells, and illustrates the possibility of achieving different effects with PBPC according to the way in which they are mobilized and collected.

**SUMMARY**

Hemopoietic progenitors circulate in human blood and can be collected by apheresis. Their

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### Table 3. Peripheral Blood Stem Cells Collected

<table>
<thead>
<tr>
<th></th>
<th>No. of Phases</th>
<th>MNC/kg (x 10^6)</th>
<th>CD34+ /kg (x 10^6)</th>
<th>CFU/kg (x 10^6)</th>
<th>LTC-IC/kg (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady state (n = 6)</td>
<td>7.5</td>
<td>10</td>
<td>ND</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Post-cyclo (n = 6)</td>
<td>7.2</td>
<td>6</td>
<td>7</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td>Post-cyclo + GM-CSF (n = 6)</td>
<td>7.0</td>
<td>6</td>
<td>15</td>
<td>134</td>
<td>22</td>
</tr>
</tbody>
</table>

**NOTE.** Results are means. Abbreviations: ND, not determined; MNC, mononuclear cells; cyclo, cyclophosphamide.

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### Table 4. Speed of Engraftment and Peripheral Blood Stem Cell Collection Characteristics

<table>
<thead>
<tr>
<th>Time to Engraftment</th>
<th>n</th>
<th>Days post-cyclo to</th>
<th>CFU/kg (x 10^6)</th>
<th>LTC-IC/kg (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤Day 15</td>
<td>6</td>
<td>(1st)</td>
<td>19 (17-21)</td>
<td>140 (18-360)</td>
</tr>
<tr>
<td>Day 16-25</td>
<td>4</td>
<td>2</td>
<td>18 (17, 18)</td>
<td>19 (9-41)</td>
</tr>
<tr>
<td>Platelets &gt;day 25</td>
<td>4</td>
<td>(1st)</td>
<td>23 (17-26)</td>
<td>35 (1.7-70)</td>
</tr>
<tr>
<td>WBC ± platelets &gt;day 25</td>
<td>4</td>
<td>(2nd)</td>
<td>36 (25, 44)</td>
<td>8.4 (1.0-12)</td>
</tr>
</tbody>
</table>

**NOTE.** Results are means (range).

* Steady-state collections.
concentration in peripheral blood can be increased by a variety of maneuvers including cytotoxic chemotherapy or growth factors such as GM-CSF or G-CSF. The quality of the cells collected depends on factors such as the previous chemotherapy or radiotherapy received by the patient and the technique used to mobilize the cells. The cells responsible for the very rapid count recovery that is typically seen after PBPC transplants appear to be relatively mature, lineage-committed progenitors, and the number of such CFU in PBPC collections generally predicts speed of engraftment. The presence of true "stem cells" in PBPC collections from man has not been formally demonstrated. However, their presence in the blood of animals and the finding of LTC-IC, primitive hematopoietic progenitors of CFU, in human blood suggest that marrow-repopulating cells are likely to be present in the peripheral circulation in man, as well.

FUTURE DIRECTIONS

The usefulness of new growth factors for PBPC mobilization, alone and in combination, is being explored by many investigators. Combinations of factors that prove too toxic or impractical to use in vivo may be used more effectively for ex vivo expansion of progenitor numbers. The possibility of autologous PBPC transplants could make the recruitment and management of unrelated stem cell donors more successful by eliminating the need for the general anesthetic required for marrow harvests.

Ultimately, it is hoped that the use of PBPC transplants will decrease the morbidity and mortality associated with the hematologic toxicity seen with aggressive therapy for malignant disease. Reduction of such toxicity should allow dose escalation of cytotoxic therapy for patients with drug-sensitive cancer, hopefully translating into improved cure rates for such tumors.

Finally, PBPC may prove to be convenient targets for genetic modification either to generate progenitors as vehicles to deliver immunomodulatory molecules such as cytokines or growth factors to human tumors or metastases, or as targets for gene therapy for inherited human disorders.

The usefulness of PBPC for hematopoietic support has been clearly demonstrated over the last several years. The next few years should define the extent to which this role will expand in clinical medicine.

ACKNOWLEDGMENT

These studies were approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. Informed consent was obtained from all patients and volunteer blood donors participating in these studies.

REFERENCES

Discussion

Dr. Winkelstein: We have been using G-CSF to prime peripheral blood and can verify your observation of very rapid recovery of platelet counts. Most of our patients have recovered platelet counts within 20 days after myeloablative therapy using G-CSF-primed peripheral stem cells alone. They often get through with one or no platelet transfusions, which is quite impressive when compared with autologous bone marrow. My questions deal with some other markers of the LTC-IC. Other investigators have proposed that two other markers, CD38 and CD33, might be useful. You commented on the DR composition of these cells and the rhodamine. Do you have any information as to whether they are CD33 or CD38 positive or negative, and do you think this is relevant?

Dr. Hoggie: They are negative for CD33 in our hands. Other investigators have looked at CD34+ subpopulations in the peripheral blood. Investigators from Milan have mobilized peripheral blood stem cells with GM-CSF. Most of the CD34+ cells that were mobilized also appeared to be CD33+, but some were CD33-. These investigators reported that enumerating total CD34+ cells has predictive value in assessing the quality of the graft.

Dr. Winkelstein: In our laboratory, using three-color analysis of these cells, we find that about 4% of the CD34+ cells are CD38- and DR-, so it is a very small percentage. In most cases these are mobilizable cells.

On another point, it seemed that most of your data on the numbers of colony-forming cells referred to chemotherapy plus GM-CSF. Do you have any information about the growth factors alone, and how much of an expansion of the culture-forming cells in the peripheral blood pool these give?

Dr. Hoggie: We have not used the growth factors alone. Others have and clearly see expansion, but in the experience of most people, there is at least an additive effect of combining some kind of chemotherapy with the growth factors.

Dr. Bergstrom: My question about the LTC-IC relates to Dr. Winkelstein's question. Irv Weissman says that a stem cell is a stem cell is a stem cell. Is an LTC-IC an LTC-IC? How much heterogeneity or homogeneity do you see in that group? Do you think there are separate populations, maybe some that are not relevant to transplant at all, that you are including?

Dr. Hoggie: An LTC-IC is defined by the assay. As you can see from the in vitro data I presented, there is heterogeneity among LTC-IC in terms of their proliferative and differentiative capacity. So my feeling is that there may indeed be heterogeneity in this population in their in vivo behavior as well, and that we may find out that they do not all have stem cell potential.

Dr. Bergstrom: In your peripheral blood work have you ever documented that you have increased the number of LTC-IC over time, outside the body, using ex vivo type of treatments?

Dr. Hoggie: No, we have not. We have tried a number of different combinations of growth factors. If you maintain bone marrow cells in LTC, the number of LTC-IC gradually declines over time. When you add growth factors to try to reverse that decline, you can enhance the maintenance somewhat, but we have never achieved net expansion of LTC-IC. Other investigators, for example Steve Emerson, say they have achieved that kind of expansion in slightly different systems.

Dr. Pahwa: Why do you get increased CD34+ cells in peripheral blood after priming with G-CSF, because we know that G-CSF works in terminal differentiation and not in the progenitors—so why do you get so much CD34+ in the peripheral stem cells?

Dr. Hoggie: You are correct in saying that G-CSF acts on terminal granulocyte differentiation, but in our in vitro studies in LTC, we have seen effects of G-CSF on much earlier cell types. In fact, the combination of growth factors that we find most successful in maintaining LTC-IC in culture is a mixture that includes steel factor, interleukin-3, and G-CSF.

Dr. Pahwa: Do you think there is an interaction of G-CSF with other growth factors? Is there a whole cascade of factors that are pushing CD34 cells?

Dr. Hoggie: Well, certainly. Even if you just give one growth factor to a person clinically, there are all sorts of endogenous factors that are going to interact with that factor.

Dr. Gullati: With some of the data that has been
shown, the dosages of drug were not truly myeloablative, and I think people might want to keep this in mind. On another point, what is your feeling about the contribution of previous chemotherapy? Do you think some of the variation is from the burnout of the bone marrow, or is it true enhancement?

Dr. Hogge: It is because of the first point that you raise that I did not try to make any strong claims for the long-term reconstitutive ability of peripheral blood cells. In any kind of autologous transplant setting, unless you do gene-marking studies, for example, it is really difficult to prove that endogenous recovery is not occurring and maintaining hemopoiesis long term. With regard to your second point, our experience and the experience of a variety of investigators is that it is more difficult to mobilize cells from patients who have had a lot of previous chemotherapy. If you count LTC-IC in bone marrow samples, the concentration is only modestly decreased in patients who have received previous chemotherapy as compared with those who have not. However, certainly the clinical experience is that patients heavily treated with chemotherapy behave as though they have had significant reduction of their “stem cell reserve” when PBPC mobilization is attempted.
In vitro collection and posttransfusion engraftment characteristics of MNCs obtained by using a new separator for autologous PBPC transplantation


**BACKGROUND:** A clinical study was performed to evaluate the peripheral blood progenitor cell (PBPC) collection, transfusion, and engraftment characteristics associated with use of a blood cell separator (Amicus, Baxter Healthcare).

**STUDY DESIGN AND METHODS:** Oncology patients (n = 31) scheduled for an autologous PBPC transplant following myeloablative therapy were studied. PBPCs were mobilized by a variety of chemotherapeutic regimens and the use of G-CSF. As no prior studies evaluated whether PBPCs collected on the Amicus separator would be viable after transfusion, to ensure patient safety, PBPCs were first collected on another cell separator (CS-3000 Plus, Baxter) and stored as backup. The day after the CS-3000 Plus collections were completed, PBPC collections intended for transfusion were performed using the Amicus instrument. For each transplant, >2.5 x 10^6 CD34+ PBPCs per kg of body weight were transfused.

**RESULTS:** Clinical data collected on the donors immediately before and after PBPC collection with the Amicus device were comparable to donor data similarly obtained for the CS-3000 Plus collections. While the number of CD34+ cells and the RBC volume in the collected products were equivalent for the two devices, the platelet content of the Amicus collections was significantly lower than that of the CS-3000 Plus collections (4.35 x 10^10 platelets/bag vs. 8.61 x 10^10 platelets/bag, p<0.05). Collection efficiencies for CD34+ cells were 64 ± 23 percent for the Amicus device and 43 ± 14 percent for the CS-3000 Plus device (p<0.05). The mean time to engraftment for cells collected via the Amicus device was 8.7 ± 0.7 days for >500 PMNs per μL and 9.7 ± 1.5 days to attain a platelet count of >20,000 per μL—equivalent to data in the literature. No CS-3000 Plus backup cells were transfused and no serious adverse events attributable to the Amicus device were encountered.

**CONCLUSIONS:** The mean Amicus CD34+ cell collection efficiency was better (p<0.05) than that of the CS-3000 Plus collection. Short-term engraftment was durable. The PBPCs collected with the Amicus separator are safe and effective for use for autologous transplant patients requiring PBPC rescue from high-dose myeloablative chemotherapy.

**ABBREVIATION:** PBPC(s) = peripheral blood progenitor cell(s).

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going autologous hematopoietic reconstitution. It included
time to engraftment and, to the degree possible in an
autologous transplant, short-term durability of the hematopoietic graft. To ensure that the Amicus device was suitable for
clinical use, each donor/patient was monitored to en-
sure the lack of any serious adverse events during the col-
collection, transfusion, and posttransfusion engraftment period.

MATERIALS AND METHODS

Informed consent
Written informed consent approved by the Yale University
Human Investigation Committee was obtained from all
patients who met Yale University School of Medicine and
Yale Cancer Center protocol entry criteria.

Patient criteria
Patients acceptable for entry into the study were males or
non-pregnant females who were 18 years or older. Histo-
logic confirmation of malignancy was required, as was a
Karnofsky score of >80 percent and a hematocrit of >20
percent prior to study entry. Exclusion criteria included:
- The absence of a signed informed consent
- Impaired hepatic function as evidenced by a total biliru-
bin of >2.5 mg per dL
- Renal dysfunction with a serum creatinine of >2.0 mg per
dL
- A positive transfusion-transmitted disease marker
- Active bacterial or fungal infections at the time of study
entry
- Altered mental status
- Pregnancy or lactation in females.
Except for two HCV antibody-positive patients, all patients
enrolled in the protocol were negative for anti-HIV-1/2,
anti-HCV, anti-HBC, anti-HTLV-I/II, HBsAg, HIV p24 Ag, and
a serologic test for syphilis. Protocol waivers on the two
HCV-positive patients were obtained from the study spon-
sor and from the two patients. These two patients were
entered into the protocol when their oncologists requested
that they be considered under provisions of the Americans
with Disabilities Act. Appropriate administrative approvals
were obtained to permit these patients to enter the study.

Patient demographics
Of the 31 patients studied, 14 were male and 17 were female.
Their diagnoses were breast cancer (n = 8; multiple myeloma
(n = 8); non-Hodgkin’s lymphoma (n = 10); Hodgkin’s disease
(n = 4); and ovarian cancer (n = 1). The average age was 46 ±
14 years for males, and 50 ± 8 years for females. For males,
the mean height was 182 ± 9 cm and for females 162 ± 9 cm.
Weight in kg was 89 ± 16 for males and 69 ± 12 for females.

Protocol design
There were four study goals, three in vivo and one in vitro.
The goals were to: 1) demonstrate that collection of pro-
genitor cells from the autologous donor/patient using the
Amicus separator was safe; 2) show that the CD34+ PBPCs
engrafted within the time frame established in the litera-
ture; 3) demonstrate that short-term engraftment was dura-
able; and 4) determine if one could be 95 percent confident
that at least 99 percent of the PBPC products would have
platelet counts less than 4 × 10^4. The study population size
was chosen on the basis of Baxter calculations suggesting
that a sample size of at least 30 patients allows a statement
with a 95 percent confidence that at least 99 percent of the
platelet counts in Amicus-collected PBPC products would
be less than 4 × 10^4 per unit. The platelet count parameter
was used to determine sample size because a low platelet
count was necessary to ensure that the collected product
could be processed efficiently on the cell selection device
(Isolex, Nexell, Irvine, CA). Evaluation of the Isolex device,
however, was not a part of this protocol. Thus, although
CD34+ cell count was important, the platelet count was also
considered to be of great importance. The CD34+ cell count
in the Amicus collection bag was compared with the CD34+
cell count in the back-up system (CS-3000 Plus, Baxter
Healthcare) collection bag, and the number of collections
required to achieve an engraftable dose was comparable.

While all practicing oncologists at Yale were eligible to
ter refer patients, patients entered into the protocol were
referred from one of three oncologists. Pre-study chemothera-
py varied with the patient. G-CSF was started 24-72 hours af-
after the mobilizing chemotherapy was given. The G-CSF
was continued daily at a dose of 5-10 μg per kg subcutaneously
until the end of the apheresis collections. A dose of 5 μg per
kg was used for eight breast cancer patients and 10 μg per
kg was used for all other patients as per the orders of the
referring oncologist. The number of G-CSF doses admin-
istered (i.e., the number of days of G-CSF treatment during
mobilization) was individualized for each patient. After
the patient’s CD34+ cell count rose above 20 per μL, a
central venous apheresis catheter was inserted. A short-term
double-lumen 11.5 Fr × 13.5 cm hard-shelled catheter
(Mahurkar, Quinton Instrument Company, Bothwell, WA)
was preferred. The level of 20 per μL was chosen to ensure
that the patient was able to mobilize CD34+ cells into the
peripheral blood. The actual CD34+ cell counts present in
the patient at the time of apheresis varied substantially and
the mean value ±1 SD is listed in Table 1. The G-CSF
was continued at a dose of 5-10 μg per kg per day subcutaneously
until the apheresis collections had ended and the
CD34+ cell goal determined by the oncologist (usually 5-10
× 10^6 CD34+ cells/kg) had been reached. After catheter
insertion, the patients underwent a series of leukapheresis
procedures.

To ensure patient safety, the study design mandated
that the order of machine apheresis collection not be ran-
domized. The protocol called for the first engraftable dose
to be obtained using the standard PBPC leukapheresis col-
collection machine (CS-3000 Plus) used at Yale. An engraftable
TABLE 1. Patient laboratory values before and after apheresis (mean ± 1 SD)

<table>
<thead>
<tr>
<th>Device</th>
<th>WBC* (×10^9/L)</th>
<th>Hb (g/dL)</th>
<th>Hct (%)</th>
<th>Platelets (×10^9/L)</th>
<th>CD34+ cells (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amicus (n = 36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>38.5 ± 10.0</td>
<td>9.7 ± 1.2</td>
<td>28.4 ± 3.5</td>
<td>109 ± 64</td>
<td>174 ± 173</td>
</tr>
<tr>
<td>After</td>
<td>31.2 ± 13.3</td>
<td>9.8 ± 1.2</td>
<td>25.9 ± 3.5</td>
<td>89 ± 54</td>
<td>113 ± 140</td>
</tr>
<tr>
<td>CS-3000 Plus (n = 34)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>24.4 ± 14.1</td>
<td>10.0 ± 1.2</td>
<td>29.2 ± 3.6</td>
<td>96 ± 63</td>
<td>162 ± 185</td>
</tr>
<tr>
<td>After</td>
<td>22.3 ± 11.6</td>
<td>9.1 ± 1.2</td>
<td>26.8 ± 3.2</td>
<td>88 ± 65</td>
<td>141 ± 148</td>
</tr>
</tbody>
</table>

* p < 0.05

TABLE 2. Machine operational parameters (mean ± 1 SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amicus (n = 36)*</th>
<th>CS-3000 Plus (n = 34)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cycles</td>
<td>9 ± 1</td>
<td>NA†</td>
</tr>
<tr>
<td>Cycle volume (ml)</td>
<td>1167 ± 167</td>
<td>NA†</td>
</tr>
<tr>
<td>ACD (ml)</td>
<td>590 ± 122</td>
<td>683 ± 159†</td>
</tr>
<tr>
<td>Run time (minutes)</td>
<td>231 ± 271</td>
<td>164 ± 39†</td>
</tr>
<tr>
<td>WB processed (L)</td>
<td>11.9 ± 1.1</td>
<td>12.0 ± 1.9</td>
</tr>
<tr>
<td>CD34+ collection efficiency (%)</td>
<td>64 ± 233</td>
<td>43 ± 14†</td>
</tr>
</tbody>
</table>

* Number of procedures per machine. To collect the required engraftable dose of CD34+ cells/kg, some patients required >1 collection/machine. Additionally, one ovarian cancer patient and six breast cancer patients were treated using a double transplant protocol.
† Not applicable (see text).
† p < 0.05.

Apheresis collections

Machine operational parameters monitored included the number of cycles and cycle volume (Amicus device only), milliliters of ACD used, run time in minutes, liters of whole blood processed, and CD34+ cell collection efficiency (see Table 2). The CD34+ cell dose to be collected was specified by the referring oncologists. PBPCs were collected on the CS-3000 Plus device according to the following operating parameters specified by the manufacturer:

- CS-3000 Plus Interface Offset Detector setting at a mean of 110 for the collections (range 100-150)
- Whole blood flow rate setting of 70 mL per minute (range 60-80 mL/minute)
- Standard 12-15 liter volume as the amount of blood processed for each PBPC collection, small-volume collection chamber (60 mL)
- Blood citrate ratio of 12:1
- Collection of 100-150 mL of donor plasma during processing

If the collection chamber overfilled during the procedure, the bag was emptied into an open-system attached sterile bag, the small-volume collection chamber was filled with saline, and the collection resumed. A calcium gluconate drip was used if the patient complained of symptoms of citrate toxicity during the procedure.

The Amicus instrument was also operated using the manufacturer's instructions. The PBPC collection is controlled via the MNC collection computer software. The Amicus device collects progenitor cells by employing a single chamber design; the collection process, however, occurs in stages. Whole blood enters through the inlet line and immediately is separated into RBCs and plasma. The plasma carries platelets out of the chamber for return to the patient (Fig. 1). For the separation stage in the Amicus centrifuge, some plasma is recirculated and an auto-elutriation process enhances separation of platelets from MNCs and reduces platelet content in the PBPC product. PMN cells exit the chamber with the high hematocrit RBCs while the MNCs remain in the chamber during each cycle. During the harvest stage of each cycle, the hematocrit of the chamber is increased to isolate the MNC layer and the position of the chamber interface is increased until the MNCs begin to exit through the plasma line (Fig. 2). Thus, the collection of progenitor cells is an intermittent process. The MNCs collected by the device are diverted into a plastic storage bag (PL2410, Baxter) integrally attached to the collection harness, and located outside the centrifuge.

Amicus machine parameters were set as follows:

- Mean of nine cycles (range, 8-12)
- 1000 mL cycle volume
Fig. 1. Platelet collection. Platelet-rich plasma (PRP) is separated from the RBCs and carried out of the chamber. High-efficiency platelet separation occurs due to plasma recirculation and auto-elutriation.

Fig. 2. White cell collection. PMNs settle to the high-G wall and exit with RBCs. MNCs initially settle toward the high-G wall but eventually float up to the interface. They are then drawn back toward the low-hematocrit inlet region where they resettle again toward the high-G wall.

- RBC offset value of 6.5-7.5
- Interface set point of 0.60
- A flow rate of 60-80 mL per minute
- A citrate transfusion rate of 1.5 mg per kg per minute
  (12:1 ratio of blood to citrate)

The procedure included collection of 100-150 mL of donor plasma.

Patient parameters
Donor/patient samples were collected from the apheresis catheters immediately before and after the collection. Patient data included: hemoglobin, hematocrit, WBC count, and platelet count (Coulter STK-S, Raritan, NJ; Hialeah, FL), and CD34+ cell count. CD34+ cell samples were incubated with 20 μL of CD34+ reagent and with 20 μL of control reagent to assess the amount of nonspecific antibody binding. Both circulating CD34+ cells and CD34+ cells in the collected apheresis products were processed and counted the same way with the exception that product samples with a WBC count >50,000/μL were diluted as necessary. The number of CD34+ cells present were counted on a flow cytometer (FacsCalibur using the ProCOUNT Progenitor Cell Enumeration Kit, Becton Dickinson Immunocytometry Systems, San Jose, CA). This technology is FDA-approved and the technique directly references the Sutherland method in the guidelines of the International Society of Hemotherapy and Graft Engineering.

Adverse event monitoring
Patients were monitored for adverse events during the apheresis collections. Adverse events were categorized as mild, moderate, and severe (see Table 3). For circulatory or limb paresthesias, mild events were defined as those that resolved with administration of oral calcium supplements. Moderate paresthesias, with or without muscle cramps, were those that required interruption of the procedure in conjunction with use of oral calcium supplements. Severe paresthesias were categorized as those that failed to respond to the above-mentioned therapies and required treatment with intravenous calcium and magnesium supplements. For sneezing, mild reactions were those that required no therapy; moderate to severe symptoms were those treated with antihistamines. Headaches were considered mild or moderate if they resolved with oral administration of acetaminophen. Severe headaches were those that failed to resolve with analgesics and required medical consultation by a physician. Chills were mild or moderate if they responded to warming blankets. Chills were severe when patients showed evidence of true rigors that failed to respond to conservative therapy, resulting in cessation of the procedure in addition to medical assessment. Mild or moderate nausea required brief interruption of the procedure and use of anti-emetics. Severe nausea required anti-emetics, cessation of the procedure, and medical assessment by a physician.

PBPC product analysis
Analysis of the PBPC product collected included measurement of volume of RBCs collected, platelet count \( \times 10^9 \) per bag, granulocyte and MNC counts \( \times 10^8 \) per bag, and CD34+ cell counts \( \times 10^7 \) per bag. Hematology assays and CD34+ cell counts were performed as described above. The PBPC volume was determined by weighing the full bags, subtracting the tare weight of the empty bag, dividing by the specific gravity of the progenitor cell product (1.050), and calculating the volume of the progenitor cell product.

The instrument CD34+ cell collection efficiency was calculated according to the formula:

\[
\text{CD34+ Efficiency (\%)} = \frac{\text{Total CD34+ Yield}}}{} 
\times 100
\]

\[
\text{CD34+ Yield} = \frac{\left(\frac{\text{Donor Pre-CD34+} - \text{CD34+/μL} \times \text{Whole Blood (μL)} \times 1000}{2}\right) - \frac{\text{Donor Post-CD34+} - \text{CD34+/μL} \times \text{ACD (μL)} \times 1000}{2}}{\text{Whole Blood (μL)}}
\]

Transfusion protocol
Most PBPC transfusions were performed on an outpatient basis. Patients generally completed high-dose chemotherapy at least 24 hours before transfusion of PBPCs, but
the standard conditioning regimens varied and were disease-dependent. All patients received only autologous cells collected via the Amicus device (>2.5 × 10^8/kg). On the day of transfusion, the frozen cells were placed in a plastic overwrap bag and thawed at the bedside in a 37°C waterbath. Within 15 minutes of thawing, PBPCs were transfused over 20 to 30 minutes through a 260-micron standard blood filter; the filter was flushed with saline to ensure that all cells were transfused. All patients were given hydration therapy, were premedicated with 50 mg diphenhydramine and 650 mg acetaminophen, and were monitored during the transfusion. Following transfusion and subsequent hydration, asymptomatic patients were discharged from the clinic once their urine became heme-negative by dipstick. The patients took prophylactic oral antibiotics and monitored themselves for fever. Patients received G-CSF 5 to 10 μg per kg per day subcutaneously, after the transfusion until the total WBC count exceeded 5000 per μL. Patients were generally seen daily in the clinic until they achieved engraftment. Patients with <500 granulocytes per μL and a temperature over 101°F were admitted to the hospital with a diagnosis of neutropenic fever and given intravenous antibiotics. RBC transfusions were given when the hemoglobin fell below 8 g per dl or the patient became symptomatic; platelets were given for a count below 20,000 per μL or if bleeding occurred. These criteria are within the Yale institutional guidelines for blood component usage. Because the Yale guidelines in place during the study recommended a transfusion threshold of <20,000 per μL for platelet transfusions, and the engraftment criterion for platelets was >20,000 per μL, 100 percent of the platelets transfused met institutional guidelines. All patients were monitored for time to engraftment and for long-term follow-up. WBC engraftment was defined at an absolute neutrophil count of >500 per μL and platelet engraftment was defined as being the first of three consecutive days with the platelet count >20,000 per μL, unsupported by platelet transfusion.

Statistical analysis

Results were analyzed and expressed as mean ± 1 SD. Comparisons between machines or collections were performed using a two-tailed, paired or unpaired t test, as appropriate for the comparisons being made. Significance was taken to be p<0.05.

TABLE 3. Adverse collection events

<table>
<thead>
<tr>
<th></th>
<th>Amicus (total procedures = 36)</th>
<th>CS-3000 Plus (total procedures = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>Circumoral paresthesias</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Limb paresthesias</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Chills</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Muscle cramps</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sneezing</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nausea</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Headache</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

RESULTS

Apheresis machine operational parameters

The two instruments used similar amounts of ACD and processed equivalent volumes of whole blood (Table 2). The Amicus device (run time, 231 minutes) took an average of 47 minutes longer to process the same 11.9 L as did the CS-3000 Plus machine (run time of 184 minutes, p<0.05). However, the collection efficiency was statistically greater for the Amicus device (64 ± 23 percent) than for the CS-3000 Plus device (43 ± 14 percent, p<0.05).

Apheresis collections

As mentioned above, except in two patients, the CS-3000 Plus device was chosen for initial collection of the back-up transfusable dose for purposes of ensuring patient safety. Thus, the Amicus collections were obtained over the subsequent 1-2 days depending on the size of the dose transfused. The starting hematocrit/hemoglobin, platelet count, and number of CD34+ cells per μL were equivalent for the donors undergoing the Amicus collections vs. those undergoing collection via the CS-3000 Plus (Table 1). However, the starting WBC counts were higher for the Amicus collections (p<0.05) because the patients had received an additional 1-2 injections of G-CSF and more time had elapsed since the myelo-suppressive chemotherapy. Adverse reactions related to the donation process were few in number and clinically mild to moderate. During collection, no serious adverse donor events attributable to either of the two separators were seen (see Table 3).

PBPC product analysis

There were differences in the composition of the PBPC products prepared by the two instruments (Table 4). PBPCs collected with the Amicus instrument had a hemoglobin level of 2.2 g per dl in 241 mL. PBPCs collected with the CS-3000 Plus instrument had a hemoglobin level of 6 g per dl in 78 mL. When adjusted for volume, however, the hemoglobin values were 5.2 g per bag for the Amicus collections and 4.9 g per bag for the CS-3000 Plus collections (p<0.05). A significantly lower platelet count was noted for the Amicus PBPC collection (4.4 ± 2.4 × 10^9 platelets/bag) versus the CS-3000 Plus collection (6.6 ± 6.3 × 10^9 platelets/bag, p<0.05). The number of granulocytes and MNCs collected by the Amicus separator was greater than that collected by the CS-3000 Plus separator (p<0.05), but the number of CD34+ progenitor cells × 10^9 per bag collected with the two
TABLE 4. Apheresis PBPC product laboratory values (mean ± 1 SD)

<table>
<thead>
<tr>
<th></th>
<th>Volume* (mL)</th>
<th>Hb (g/dL)</th>
<th>Platelets* (×10^9/L)</th>
<th>PMN* (×10^9/L)</th>
<th>MNC* (×10^9/L)</th>
<th>CD34+ cells† (×10^6/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amicus (n = 36)</td>
<td>241 ± 52</td>
<td>5.2 ± 3.5</td>
<td>4.4 ± 2.4</td>
<td>1.93 ± 1.1</td>
<td>1.9 ± 0.9</td>
<td>783 ± 635</td>
</tr>
<tr>
<td>CS-3000 Plus (n = 34)</td>
<td>78 ± 28</td>
<td>4.9 ± 2.2</td>
<td>6.6 ± 6.3</td>
<td>1.07 ± 1.4</td>
<td>1.4 ± 0.8</td>
<td>888 ± 793</td>
</tr>
</tbody>
</table>

* p<0.05.
† CD34+ cells collected per kg donor body weight (p>0.05).

Transfusion results
The mean dose of CD34+ cells transfused was 8.03 ± 6.50 × 10^6 per kg (Table 5). The range of CD34+ cells transfused into the enrolled study patients varied. A total of four patients received a dose of 2.5 to 3.5 × 10^6 CD34+ cells/kg; five received a dose of 3.6 to 4.6 × 10^6 per kg; 13 patients received 4.7 to 7.0 × 10^6 per kg; seven received 7.1 to 12.0 × 10^6 per kg; four received 12.1 to 20.0 × 10^6 per kg; and one patient received 38 × 10^6 per kg. The engraftment time needed to achieve >500 PMNs per μL for the PBPCs collected on the Amicus device was equivalent to published literature values (Table 5). Similarly, engraftment time to achieve over 20,000 platelets per μL was comparable to engraftment times published in the literature. These results are also equivalent to the neutrophil and platelet engraftment times seen by the authors (time to reach PMN > 500/μL, 9.3 days; time to reach platelet count > 20,000/μL, 10.9 days), using other cell separators (Spectra, Cobe, Lakewood CO, and CS-3000 Plus, Baxter) at Yale (n = 400).

One patient with ovarian cancer and six patients with breast cancer received double transplants (1 to 3 months apart) with PBPCs collected via the Amicus instrument. Each patient's first and second transplants engrafted within the same time frames described earlier.

While no serious adverse events directly related to the progenitor cell collection were noted, two patients did experience serious complications in the peritransplant period. Two days after transfusion of cells collected via the Amicus device, a 42-year-old male patient with a diagnosis of non-Hodgkin's lymphoma, was admitted to the hospital for shortness of breath and chest pain with a presumptive diagnosis of a pulmonary embolism; this diagnosis was never confirmed. He was treated with a standard anticoagulant regimen, however, and his symptoms resolved. He engrafted within 9 days and is currently alive 12 months after transfusion, with no evidence of disease. The other patient, a 50-year-old female with breast cancer, presented to the clinic for PBPC transfusion complaining of a swollen neck; she was diagnosed with superior vena cava syndrome. She, too, was admitted to the hospital, where her symptoms resolved with treatment. After her PBPCs were transfused, she engrafted on Day 8. This patient died 5 months later, however, of recurrent breast cancer. Both patient incidents were classified as unrelated to Amicus collection or PBPC transfusion.

For a total of 34 cell transplants, 68 RBC units and 225 random-donor platelet units were required for transfusion therapy during the preengraftment period. The mean number of RBC units transfused was 2.0 (10 patients received no units, three received 1 unit, eight received 2 units, four received 3 units, four received 4 units, and five received 5 or more units). The mean number of random-donor platelet units transfused was 6.6 (eight patients received no units, 15 received up to nine units, 10 received between 10 and 20 units, and one patient received over 20 units).

DISCUSSION
The data demonstrate that the Amicus separator is safe and effective for collection of CD34+ PBPCs. The Amicus device provided an acceptable product that reliably produced engraftment without the occurrence of serious adverse events. PMN engraftment occurred predictably by 8.7 days (PMN > 500/μL) and platelet engraftment by 9.7 days (platelets > 20,000/μL). These values are equivalent to those published in the literature, and are comparable with the authors' experience with other cell separators.

The collection of CD34+ PBPCs for use in autologous hematopoietic progenitor cell transplants has become accepted as a standard of care for some malignancies. It has essentially replaced a marrow harvest for restoration of marrow function after myeloablative chemotherapy. Refinements of apheresis collection protocols include increasing the CD34+ cell collection efficiency, decreasing the number and duration of collections, and improving purity of the final product by decreasing the number of non-CD34+ cells collected, such as:

TABLE 5. Days to PBPC engraftment (mean ± 1 SD)

<table>
<thead>
<tr>
<th>Device</th>
<th>CD34+ cells infused (×10^6/kg)</th>
<th>PMNs (×10^9/L)</th>
<th>Platelets (×10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amicus</td>
<td>(n = 33)</td>
<td>(≥500/μL)</td>
<td>(≥20,000/μL)</td>
</tr>
<tr>
<td>Literature</td>
<td>NA</td>
<td>NA</td>
<td>9.7 ± 1.5</td>
</tr>
</tbody>
</table>
| * n = 20 patient transplants followed by >50,000 platelets per μL.† See Langenmayer and Smith.
as platelets. Requiring fewer collections to achieve an effective dose would provide substantive patient benefit. It would permit earlier central venous catheter removal, and this would, in turn, decrease the risk of infection or hemorrhage due to an indwelling foreign body (catheter) in an infection-prone patient. It would also mean fewer doses of G-CSF or other hematopoietic growth factors, thus reducing side effects and costs associated with its use. Fewer collections would also result in decreased freezer storage needs and thus increase the cost-effectiveness of the treatment program.

While most other collection parameters were essentially equivalent, the length of time needed for a collection cycle on the Amicus device increased collection time on that device by 5 to 10 minutes per cycle. This could extend the collection time by as much as 60 minutes, depending on the donor/patient CD34+ cell count and WBC count. For patients with WBC counts >10,000 per µL, the cycle volume can be lowered to maximize collection efficiency; this would also shorten the time required for the overall collection. Adjustment of the elutriation and concentration aspects of the Amicus MNC collection process may permit more efficient collection for patients who mobilize poorly and who might never develop a CD34+ cell count above 30 to 40 per µL; more research is needed in this area. The Amicus device also provides more computer control and the menu-driven computer touch screen interface offers an overall more user-friendly design than does the CS-3000 Plus device. Engraftment of cells collected via the Amicus device reliably occurred in 8 to 10 days. This posttransfusion time frame is equivalent to the time expected for PBPCs collected using other cell separator devices.

The transfused dose of PBPCs collected via the Amicus equipment was always greater than 2.5 x 10^6 per kg. Some doses were much larger than this. Further studies are needed to evaluate engraftment characteristics of a lower dose of these cells such as 1.5 x 10^6 per kg, or less. It is possible that the lack of randomization necessitated by patient safety concerns affected the efficiency results. Further clinical studies of the CS-3000 Plus and the Amicus devices involving full randomization would be revealing in this regard. This manuscript reports on a clinical comparison of the Amicus and the CS-3000 Plus Instruments. No comparison data for the Amicus and other cell separators exist at this time. A comparison study of the Cobe Spectra and the Amicus separator is in progress by the authors.

The Amicus device collects CD34+ cells more efficiently, although at a slower rate than does the CS-3000 Plus device. Platelet content was lower in the Amicus collection products than in the CS-3000 Plus collection products. This is important because development of donor thrombocytopenia is an ongoing concern with a variety of cell separators and their computer programs.11,12 Because it is more efficient than the CS-3000 Plus, the Amicus separator would be more useful for collecting PBPCs from patients who are poor mobilizers and who have marginal circulating CD34+ cell counts. PBPCs collected with the Amicus device engraft in a time frame equivalent to that in the literature. Short-term engraftment durability was 100% in that no one became aplastic. Thus the Amicus Separator appears to be safe and effective for collection and transfusion of PBPCs from oncology patients undergoing progenitor cell rescue following myeloablation.

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REFERENCES

The use of short tandem repeat polymorphisms for monitoring chimerism following bone marrow transplantation: a short report

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Following immunohaematopoietic stem cell transplantation, it is of importance to determine whether the new blood forming system is of recipient or donor origin and such phenotypic characterisation is called chimerism analysis. This is a dynamic process, which may be complete, mixed or split between compartments and in this way, plays an increasingly important role in predicting outcome for engraftment, rejection or residual disease predating the need for pre-emptive immunotherapy. Based on recent workshop recommendations, peripheral blood cells have been used in the short tandem repeat (STR) assay to serially characterise the haematologic course and so evaluate the usefulness of this system. Forty-six patients from a single centre were followed serially for periods ranging between 3 and 60 months. The analysis was initially performed using the Applied BiosystemsProfiler Plus Kit; currently, the Promega Powerplex 16 system is used. The overlap between the two assays has allowed for continuous comparison. The initial analysis was performed at 14 days post-transplant and repeated monthly. Stored DNA from the patient and donor was used to establish the pre-transplant profile. All post-transplant analyses were performed using peripheral blood. The results obtained were expressed as a percentage of the donor profile. To illustrate the ability of this technology, three representative profiles are described. In the first, stable engraftment is confirmed at 20 months with only donor pattern present. The second is intermediate, and while the patient is clinically disease free, there exists stable mixed chimerism at about 75% of donor cells. The third patient initially engrafted but the reappearance of recipient alleles presaged a haematological relapse; the latter is an indication for salvage with donor lymphocyte infusion and here this assay will be used to show the effectiveness of the intervention. These preliminary results show this to be a useful additional tool in monitoring post-transplant engraftment. As a basis for pro-active therapy, a larger study integrating the results of haematological and cytogenetic markers is planned.

Keywords: unrelated marrow, allograft, chimerism, engraftment, relapse

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Introduction

Early studies recognised the importance of establishing chimerism in patients after bone marrow transplantation but investigators had to rely on techniques such as red cell phenotyping.\textsuperscript{1,2} Those techniques were limited by their lack of polymorphism and sensitivity. However, one method still of value in gender mismatched transplantation is fluorescent in situ hybridisation (FISH) for X- and Y-chromosomes.\textsuperscript{3}

In contrast, short tandem repeat (STR) polymorphism, a human identification marker initially used in forensic pathology, can be employed to monitor chimerism in post-transplant patients.\textsuperscript{4} These are repeated units of nucleotides usually two to four base pairs in length and individuals have a variable number of repeats at each locus tested, thereby ensuring his or her unique profile. The advantages of this methodology include sensitivity, reproducibility of results, small amounts of sample needed and the relative speed as well as cost effectiveness of the test.

The present study was designed to assess the use of this technology to monitor chimerism in patients following unrelated allogeneic bone marrow transplantation.

Material and methods

DNA was extracted from EDTA-preserved whole blood and bone marrow, following standard extraction methods making use of the DNAzol\textsuperscript{®} reagent supplied by Invitrogen, or Qiagen.

After quantification of the DNA yield, approximately 0.5 to 1 µl of DNA template was amplified in a single step 5 µl reaction volume, using one of two DNA profiling kits namely AmpFISTR Profiler Plus (Applied Biosystems) or PowerPlex\textsuperscript{®} 16 System (Promega). DNA amplifications were performed on the GeneAmp\textsuperscript{®} PCR system 9700 thermal cycler following the supplier protocols. Amplified products were profiled with an ABI 310 DNA sequencer using sequenced allelic ladders. Interpretation of the results was performed using Genotyper\textsuperscript{®} software.

The results are expressed as a percentage of donor profile.

Assessment of chimerism

Chimerism of post-transplant samples was assessed by comparing the pre-transplant patient DNA profile, the donor DNA profile and the post-transplant patient DNA profile.

If a ‘stutter peak’ corresponded to a donor or patient DNA allele, it was only reported if the allele represented more than 5% of the total DNA per marker system.\textsuperscript{3}

Quantification of the patient and donor DNA contribution to a mixed DNA profile was calculated semiquantitatively by summing the total peak height and peak area of all alleles per marker system and then calculating the percentage DNA contributed by each individual allele of the system. Homozygous alleles were averaged.

Results

Initially, 61 patients were enrolled in the study. Fifteen were lost to follow up. Thirty-three male and 13 female patients whose ages ranged from 5 years to 64 years and who received either related or unrelated bone marrow transplants for a variety of haematologic malignancies (see Figs 1 and 2) were monitored serially for periods ranging between 3 and 60 months.

The results of three representative profiles are shown in Tables 1, 2 and 3. These patients were transplanted with human leucocyte antigen (HLA) matched unrelated donors. The results of an acute myeloid leukaemia (AML) patient (DS) who was monitored regularly for 20 months after transplant are shown in Table 1. No chimerism was detected. Chimerism was detected in a paroxysmal nocturnal haemoglobinuria (PNH) patient (WB) at 6 months post-transplant. The percentage of donor cells present was calculated and has remained at approximately 75% for 21 months (Table 2). The third representative profile, shown in Table 3, is that of a chronic myeloid leukaemia (CML) patient (MA). The

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td></td>
<td>Pre Tx* Donor</td>
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<td>PentE</td>
<td>14: 16</td>
</tr>
<tr>
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<td>8: 11</td>
</tr>
<tr>
<td>D7S930</td>
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<td>9: 13</td>
</tr>
<tr>
<td>CSF1PO</td>
<td>9: 12</td>
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<tr>
<td>PentaD</td>
<td>11: 12</td>
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<tr>
<td>Amelogenin</td>
<td>34: 34 34: 34</td>
</tr>
<tr>
<td>% Donor</td>
<td>100% 100% 100%</td>
</tr>
</tbody>
</table>

Patient DS; diagnosis: AML
* Patient's pre-transplant STR profile.

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results showed the reappearance of patient profile at 4 months post-transplant. Medication was altered and a 100% donor profile reappeared at 11 months. At 17 months, the patient profile reappeared indicating a need for intervention once again.

**Comment**

Allogeneic immunohaematopoietic stem cell transplantation is an established therapeutic option in an increasing number of diseases ranging from aplasia through severe combined immunodeficiency or

**Table 2 Stable chimerism**

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<tr>
<th></th>
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<td>11; 12</td>
<td>11; 12; 13°</td>
<td>11; 12; 13°</td>
<td>11; 12; 13°</td>
</tr>
<tr>
<td>D7S820</td>
<td>9; 12</td>
<td>9; 11</td>
<td>9; 11; 12°</td>
<td>9; 11</td>
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<td>13; 14</td>
<td>12; 13; 14</td>
<td>13; 14; 12°</td>
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<tr>
<td>vWA</td>
<td>18; 19</td>
<td>15; 17</td>
<td>15; 17; 16°; 19°</td>
<td>15; 17; 16°; 19°</td>
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<td>21; 19°</td>
<td>21; 19°</td>
<td>21; 19°</td>
</tr>
<tr>
<td>Amelogenin</td>
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<td>22; 34</td>
<td>22; 34</td>
<td>22; 34</td>
<td>22; 34</td>
</tr>
<tr>
<td>% Donor</td>
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<td>75%</td>
<td>75%</td>
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Patient WB, diagnosis: PNH. *Patient’s pre-transplant STR profile; °re-emerging patient alleles, post-transplant.

**Table 3 Increasing chimerism**

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<th>Pre Tx*</th>
<th>Donor</th>
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<th>11 months</th>
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<td>15°; 17; 19</td>
<td>17; 19</td>
<td>15°; 17; 19</td>
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<tr>
<td>THO1</td>
<td>8; 9</td>
<td>9; 3</td>
<td>8; 9</td>
<td>9; 3</td>
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<td>29; 32°</td>
<td>29; 30</td>
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<td>16; 17</td>
<td>16; 17</td>
<td>16; 17; 19°</td>
<td>16; 17</td>
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<td>34; 34</td>
<td>34; 34</td>
<td>22°; 34</td>
<td>34; 34</td>
<td>22°; 34</td>
</tr>
<tr>
<td>% Donor</td>
<td>100%</td>
<td>88%</td>
<td>100%</td>
<td>88%</td>
<td>100%</td>
<td>79%</td>
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Patient’s pre-transplant STR profile; °re-emerging patient alleles, post-transplant.
haemoglobinopathies to both benign and haematologic diseases or solid tumours. Post-transplantation monitoring is used to predict negative events such as graft failure or rejection, disease relapse or graft-versus-host disease. In this context, haematopoietic chimerism is of value and in addition, this assists detection of minimal residual disease. The frequency with which studies should be done varies, with indication being monthly for secondary graft failure.

In the present study, the applicability of molecular techniques was serially evaluated and our experience adds to that previously reported from other centres.

There is particular interest in the post-transplant data both for patients transplanted with matched unrelated donors and reduced intensity conditioning with modifications consequent upon prophylactic donor lymphocyte infusion. Finally, these measures allow exploration of some of the newer interests, including cell transfer in mesenchymal transplants and circulating endothelial progenitors.

Summary and conclusion
It is concluded that this methodology is of established value in clinical transplantation to monitor engraftment status and expose early disease relapse.

Acknowledgements
Supported by the Haematological Research & Educational Trust and the Chairman’s Fund of the Anglo-American Corporation with grants from The Anthony Taberer, Louis Shill and Margaret Ward Foundations. Christine Dölling helped with the bibliographic review and Sharon Griffin typed the manuscript. Thanks to both research assistants. Each contributed equally to all facets of this manuscript, including the intellectual design of the study, collection of the data presented and writing of this draft.

References
1 Khan F, Agarwal A, Agarwal S. Significance of chimerism in haematopoietic stem cell transplantation: new variations on an old theme. Bone Marrow Transplant 2006; 34: 1–12.
8 Belon J, Halaburda K, Bieniaszewska M, Reichert M, Bieniaszewski L, Piekarska A et al. Early complete donor haematopoietic chimerism in peripheral blood indicates the risk of
Use of short tandem repeat polymorphisms for monitoring chimerism following bone marrow transplantation

Minimal number of circulating CD34+ cells to ensure successful leukapheresis and engraftment in autologous peripheral blood progenitor cell transplantation


BACKGROUND: The number of peripheral blood (PB) CD34+ cells has been widely used to monitor the timing of leukapheresis for autologous transplantation. However, no cutoff value for CD34+ cells in PB has been defined as a guideline for the identification of patients in whom the harvest would be effective and those in whom there was a high probability of failure.

STUDY DESIGN AND METHODS: The present study investigated the best threshold of CD34+ cells in PB for successful harvesting and engraftment, using 263 PB samples with their corresponding leukapheresis components. In addition, that measure has been compared to other commonly used criteria such as the white cell count, the number of mononuclear cells, and the number of colony-forming units–granulocyte macrophage in PB.

RESULTS: Time to engraftment of both granulocytes and platelets was significantly influenced by the number of CD34+ cells transfused, but all patients receiving \( \geq 0.75 \times 10^6 \) CD34+ cells per kg achieved engraftment within a reasonable number of days (>0.5 x 10/L granulocytes by Day 11 and >20 x 10/L platelets by Day 13). A clear correlation between the number of CD34+ cells per µL in PB and of CD34+ cells per kg collected was found at each apheresis (\( r = 0.9, p<0.0001 \)). Moreover, the number of CD34+ cells per µL measured in PB the day the first leukapheresis was initiated displayed an excellent correlation with the total amount of CD34+ cells per kg finally collected (\( r = 0.81, p<0.0001 \)). On the basis of the regression curve obtained and the clinical engraftment results, it was found that the presence of \( >5 \) CD34+ cells per µL in PB ensured a good yield from the harvest in 95 percent of patients and would avoid an unsuccessful harvest in 81 percent of cases.

CONCLUSION: A dose of only 0.75 x 10^6 CD34+ cells per kg guarantees hematopoetic recovery within a reasonable number of days. To initiate a leukapheresis from which enough progenitor cells may confidently be obtained, a minimum of 5 CD34+ cells per µL in PB is required.

Peripheral blood progenitor cells (PBPCs) have become the most widely used source of stem cells with which to rescue patients undergoing myeloablative therapy. Most transplant groups have established their own minimal number of CD34+ progenitor cells required within the harvest to ensure a rapid and permanent engraftment. Although these figures vary among centers, depending on their internal control values, in most studies the speed of recovery correlates with the number of CD34+ cells and of colony-forming units–granulocyte-macrophage (CFU–GM) transfused.15 Because this value is apparently critical to predicting engraftment, an obvious question is how to predict the number of progenitor cells expected from the harvest. For this purpose, different peripheral blood (PB) values have been used to reach a decision on when to initiate apheresis; the most commonly used are the white cell (WBC) count, the number of mononuclear cells (MNCs), the number of circulating CD34+ cells, and the number of CFU–GM.6,7 In particular, these last two values have been shown to correlate with the amount of progenitor cells finally collected in the harvest component.8,9 The measurement of CFU–GM in PB is not practical in clinical decision making, as results are obtained only after 14 days of culture. On the other hand, the estimation of the circulating CD34+ cells offers three major advantages: 1) results are obtained within a few hours; 2) it has high intralaboratory reproducibility; and 3) there is permanent documentation of the results.10 Accordingly, at present, most groups use this value to monitor the timing of

ABBREVIATIONS: CFU–GM = colony-forming units–granulocyte-macrophage; MNCs = mononuclear cells; PB = peripheral blood; PBPC(s) = peripheral blood progenitor cell(s); WBC(s) = white cell(s).

From the Department of Hematology, University Hospital, Salamanca, Spain.

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Volume 38, April 1998 TRANSFUSION 385
Mobilization and harvesting of peripheral blood stem cells.

Moog R.

Institute for Transfusion Medicine, University Clinics Essen, Germany. rainer.moog@uni-essen.de

The use of peripheral blood stem cells (PBSC) as a source of hematopoietic stem cells is steadily increasing and has nearly supplanted bone marrow. The present article reviews mobilization and collection of PBSC as well as its side effects. Specialized harvesting strategies such as large volume leukapheresis (LVL) and pediatric PBSC collection are included in this overview. Under steady state conditions, less than 0.05% of the white blood cells (WBC) are CD34+ cells. Chemotherapy results in a 5-15-fold increase of PBSC. Combining chemotherapy and growth factors increases CD34+ cells up to 6% of WBC. In the allogeneic setting, granulocyte-colony stimulating factor is used alone for PBSC mobilization. Several factors affect the mobilization of PBSC: age, gender, type of growth factor, dose of the growth factor and in the autologous setting, patient's diagnosis, chemotherapy regimen and number of previous chemotherapy cycles or radiation. Harvesting of PBSC can be performed with various blood cell separators using continuous or discontinuous flow technique. Continuous flow separators allow the processing of more blood compared with intermittent flow devices resulting in higher yields of CD34+ cells for transplantation. LVL can be used to increase the CD34+ yield in patients with low CD34+ pre-counts. Processing of more blood in LVL is achieved by an increase of the blood flow rate and an altered anticoagulation regimen. Specialized strategies were developed for pediatric PBSC collection considering the main limiting factors, extracorporeal volume and vascular access. Adverse events in PBSC collection can be subdivided in apheresis associated and mobilization associated side effects. Citrate reactions due to hypocalcemia are frequent during apheresis, especially in pediatric PBSC collection and LVL. Thrombocytopenia is often observed in patients after termination of apheresis due to platelet loss during PBSC harvesting. Muscle and bone pain are frequent adverse events in allogeneic stem cell mobilization but are usually tolerated under the use of analgesics. Spleen enlargement followed by rupture is a serious complication in allogeneic donors.

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Peripheral blood stem cells

Mobilized blood cells vs bone marrow harvest: experience compared in 171 donors with particular reference to pain and fatigue

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1Alberta Bone Marrow Transplant Program and Departments of Medicine, Nursing and Epidemiology, Tom Baker Cancer Centre, Calgary, Alberta, Canada; and 2Alberta Bone Marrow Transplant Program and Departments of Medicine and Nursing, Foothills Hospital, Calgary, Alberta, Canada

Summary:

This prospective study compared the donor experience of blood cell (BC) mobilization and leukapheresis (n = 116) with that of bone marrow (BM) harvest (n = 55). Internal jugular catheters were inserted electively in 89% of BC donors. Most (80%) BM donors had a harvest with general anesthesia; 20% had epidural or spinal anesthesia. Pain and fatigue were frequent with both procedures and were compared in responses to questionnaires. A total of 85% of BM donors reported moderate or severe pain compared with 68% of BC donors (P = 0.02). The median duration of pain was 14 days for BM donors compared with 3 days after BC mobilization (P < 0.0001). More BM donors had pain for more than 7 days (75% vs 0%, P < 0.0001). Severe fatigue was experienced by more BM donors (49% vs 16%, P < 0.0001). Fatigue lasted significantly longer in BM donors (median 11 vs 4 days, P < 0.0001) and more BM donors were fatigued for more than 1 week (60% vs 0%, P < 0.0001). A total of 11 donors had both BM and BC collection; seven preferred the latter. Simply considered with respect to pain and fatigue, BC donation appears better tolerated by donors. However, there are other sequelae of both influencing the acceptability for individual donors.

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doi:10.1038/sj.bmt.1704418
Published online 2 February 2004

Keywords: blood stem cells; bone marrow; allogeneic transplant; donors; G-CSF; harvest

In recent years, there has been a substantial increase in the use of cytokine-mobilized blood cells (BCs) or peripheral blood stem cells (PBSC), as an alternative to bone marrow (BM) for allogeneic stem cell transplantation (SCT). Over 40% of the related donor SCTs in adults reported to the IBMTR in 1998–2000 were BC transplants (BCTs)1. While serious complications have been reported, the experience generally appears to be safe for the donor.2,3,4 There is limited information regarding the subjective experience of BC donors compared with those giving BM.5,6 We initiated a single center prospective study in 1993 to assess BC donor experience. In 1995, we began to compare donor experiences with the two collection procedures and to record any adverse effects in the years after BC donation.

Donors and methods

A combined total of 171 donors participated in the study. The 116 BC donors were recruited between April 1993 and November 1997. A total of 55 marrow donors were recruited from September 1995 to November 1997. No donors refused to participate in the study. Donors were from the adult outpatient service of a provincial BC and BM transplant program. The study began using only BC donors, and then expanded to compare the two donor populations. There was no randomization. In general, BM donors were giving to recipients who were unrelated or were related with chronic myelogenous leukemia (CML) or nonmalignancies.

BC donors

Table 1 gives some characteristics of the 116 BC donors and summarizes their mobilization and collection procedures. All donors had a full history and physical examination, blood counts, serum chemistry, and viral serology. Electrocardiogram and chest X-ray was done on donors over 40 years of age. A pregnancy test was performed on women of childbearing age.

The past medical history of BC donors revealed thalassemia minor (2), hemochromatosis (1), cervical cancer (1), Graves' disease treated with 131I (1), diabetes (4), and asthma (6). Cardiovascular problems included prior myocardial infarct and angioplasty (1), arrhythmias (3), hypertension (9), and valve dysfunction (3).

As a program standard, G-CSF was given as a multiple of 300 and 480 µg vials. In a preliminary dose-finding study, doses ranged between 5 and 16 µg/kg; a final daily dose of 7.5–10 µg/kg was selected.4 In all, 22 donors had less than 7.5 µg/kg, 13 had more than 10 µg/kg. Blood CD34+ cell counts were monitored daily and leukapheresis was performed on day 3 or 4, with day 0 being the day of the
first injection. Early in the study, some donors had peripheral venous access, but we shortly established a routine to insert internal jugular catheters under a local anesthetic, using radiologic guidance. The details of BC mobilization and collection are reported elsewhere. A total of 13 BC donors had a prior or subsequent BM harvest; in nine of these, BM harvest took place before the study, the other four were included in both BC and BM donor group studies. Eleven were asked if they had a preference for one of the procedures.

**BM donors**

The characteristics of BM donors are shown in Table 1. Pre-transplant screening was conducted by BC donors. The medical problems included: hypertension (I), asthma (3), arrhythmia treated with digoxin (1), and sleep apnea (I). In all, 45 donors had general anesthetic, nine had spinal anesthetic (donor request), and one donor who was 16 weeks pregnant was given an epidural anesthetic.

Marrow harvest was conducted using multiple large-bore needle punctures with no more than 10 ml aspirated from any one site. Target yields depended on the clinical circumstances, but were a minimum of $2 \times 10^4$ kg recipient weight. Donors were given a prescription for acetaminophen with Codeine (or an alternative in the event of intolerance).

In all, 17 marrow donors had autologous blood stored; 16 had the units infused back following the harvest. One unrelated donor refused to have the unit reinfused.

Donors were day surgical patients and usually discharged home in the afternoon.

**Data collected from donors**

Donors began recording information on the first day of G-CSF injections or the marrow harvest day. A checklist required donors to evaluate the degree of pain and fatigue (scored as mild, moderate or severe) and to record the presence of other symptoms, as listed in Figure 2 and Table 2. In addition, nursing records of the mobilization and collection were reviewed. Day 0 in this study refers to the first day of G-CSF injections in BC donors and the marrow harvest day in BM donors. On the day after the procedure, a nurse met with the donor and reviewed the data collected. Donors recorded symptoms until they were symptom free. A nurse phoned the donors weekly to ask about their well being until symptoms disappeared. BC donors were followed yearly by a letter or phone call; a blood count was requested at this time. Three nurses were responsible for data collection, data entry, and follow-up.

**Informed consent**

All donors and patients signed the consent forms approved by the University of Calgary Conjoint Ethics Review Board. BC donation was not an option for unrelated donors. Patients with malignant disease other than CML in chronic phase were eligible for studies of BC transplantation. One such patient had a BM transplant because of donor preference.

**Statistical analysis**

The proportion of BC or BM donors experiencing different intensities of pain and fatigue were compared using Fisher's exact test. The duration of pain and fatigue was evaluated using the Wilcoxon rank sum test. Analysis was performed using S-Plus 2000 software (Mathsoft Inc., Seattle, WA, USA).

**Results**

The frequent complications common to both procedures were bone pain and fatigue.

**Pain**

Bone pain in BM donors was at the site of the multiple needle aspirations, whereas that experienced after G-CSF in BC donors was widespread.

Bone pain was in the back in 68 BC donors, hips in 43, legs in 26, sternum in 19, generalized in 12, and other areas in the remainder. In all, 23 donors had pain in only one site; the remainder had pain in more than one site.

All except three BM donors had severe pain on the first post operative day. Although most donors (96% BM, 92% BC) experienced some discomfort, pain was more intense and lasted longer in BM donors. A total of 85% of BM donors experienced moderate/severe pain.

### Table 1: Donor characteristics

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<th>BM</th>
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</thead>
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<td>55</td>
</tr>
<tr>
<td>Male/Female</td>
<td>61/55</td>
<td>33/22</td>
</tr>
<tr>
<td>Age median (range)</td>
<td>38 (11-69)</td>
<td>38 (15-69)</td>
</tr>
<tr>
<td>Relationship to recipient</td>
<td>Parents 22, siblings 94</td>
<td>Related 20, unrelated 35</td>
</tr>
<tr>
<td>Days G-CSF, median (range)</td>
<td>4 (2-5)</td>
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</tr>
<tr>
<td>Two mobilization and collection procedures</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>&gt;1 collection per mobilization</td>
<td>2 (4 donors)</td>
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<td>Femoral then jugular</td>
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**Table 2: Minor and/or infrequent side effects of BC or BM donation**

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<th>Side effect</th>
<th>BC (n = 116)</th>
<th>%</th>
<th>BM (n = 55)</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Numbness/paraesthesia</td>
<td>2</td>
<td>2</td>
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<td>Rash</td>
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<td>7</td>
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<tr>
<td>Hypotension</td>
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<td>4</td>
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<tr>
<td>Hot flashes</td>
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<tr>
<td>Abdominal cramps</td>
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<tr>
<td>Constipation</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetany</td>
<td>1</td>
<td>1</td>
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</table>
donors reported moderate or severe pain compared with 68% of BC donors \((P=0.02, \text{ Figure 1})\), with the corresponding figures for severe pain of 32 and 20%, respectively \((P=0.12)\). Pain lasted a median of 14 days (range 2-56) in BM donors compared with 3 days (range 1-6) in BC donors \((P<0.0001, \text{ Figure 2})\). In total, 75% of BM donors had more than 1 week of pain compared with no BC donors \((P<0.0001)\).

**Fatigue**

Fatigue was commonly reported with both procedures, by 88% of BM donors and 77% of BC donors \((P=ns)\). In all, 71% of BM donors experienced moderate or severe fatigue compared with 49% of BC donors \((P=0.05, \text{ Figure 1})\). The figures for severe fatigue considered separately were 49 and 16%, respectively \((P<0.0001)\). Fatigue lasted significantly longer in BM donors, a median of 11 days (range 1-38) compared with 3 days (range 1-6) after BC donation \((P<0.0001, \text{ Figure 2})\). Altogether, 69% of BM donors had fatigue for more than 1 week compared with no BC donors \((P<0.0001)\).

**Other sequelae**

Figure 3 illustrates the frequency of symptoms with an incidence greater than 10% in one or both groups.

![Figure 1](image1.png) Distribution of maximum degree of pain and fatigue. BM represented by solid bars, M/S = moderate/severe.

![Figure 2](image2.png) Comparison of duration of pain and fatigue.

Headache was mostly seen in BC donors and was less severe than bone pain. Of the 74% of BC donors with headache, 53% described it as mild, 31% moderate, and 16% severe. Headache was generalized in 33 donors, frontal in 23, and occipital in 11. Some less common sequelae are summarized in Table 2.

One BC donor was found to be pregnant following the BC collection. A pregnancy test taken 3 days before G-CSF was negative. At 5 days after the collection, a home test was positive. This test result was confirmed in the laboratory. The donor was advised that the risks of G-CSF were unknown, but she decided to have an abortion largely for financial reasons.

**Blood counts**

The median precollection platelet count for BC donors was \(247 \times 10^9/\text{l} \) (range 156-467), falling to a median of \(132 \times 10^9/\text{l} \) (range 30-283) the day after collection. In 14 cases (12%), platelets fell below \(100 \times 10^9/\text{l} \) on the day after BC collection, and three fell below \(70 \times 10^9/\text{l} \) to levels of 30, 56, and \(68 \times 10^9/\text{l} \).

In BM donors, the median presurgical hemoglobin level was \(141 \text{ g/l} \) (range 107-171) and the median the day after the harvest was \(116 \text{ g/l} \) (range 85-146). In five donors, the hemoglobin count fell below 100 g/l, the lowest being 85 g/l. Platelets were unaffected in these donors, none of whom received G-CSF.

**Donors undergoing both procedures**

Of 11 donors who had both BM and BC collection, seven preferred the latter. Pain, fatigue, and recovery time were given as reasons. Of the four donors preferring BM harvest, three cited the discomfort of venous access (two jugular, one femoral). The fourth felt that too much time was required for G-CSF injections.

**Long-term follow-up**

Two BC donors refused further participation in the study as their recipients had died, and the recall for blood counts was a painful reminder. Nine donors were lost in follow-up. The remaining 105 were followed for 1-5 years (median 2).
Two BC donors developed cancer; one (aged 69) developed colon cancer, the second developed bladder and prostate cancer and is now deceased. A 34-year-old BC donor developed Guillain–Barre syndrome and another became hyperthyroid.

In all, 85 BC donors had 1–5 annual blood counts (median 2), with no significant changes compared with counts before donation.

Discussion

The trend toward the use of allogeneic BCT will doubtless continue in the light of recent randomized studies, some of which demonstrate a relative advantage of this source, at least in some patient subgroups. However, in many circumstances, BM remains an acceptable source of stem cells. The relative morbidity of BM or BCTs over the long term, particularly from chronic GVHD, has yet to be evaluated. Given the lack of demonstrable differences in the risk to donors, the issue of perceived tolerability of obtaining stem cells from BM or BC collections therefore remains important.

We found that the intensity and duration of both pain and fatigue were significantly greater in BM donors. Clearly, the nature of the pain was different for the two groups. Other effects of the procedures, either common to both or largely restricted to one or the other, were observed and are similar to other reports of BM and BC donation. We cannot evaluate the contribution of these to the tolerability of the procedures as a whole or determine their influence on the preferability of one procedure over the other.

There are some published reports available, comparing the procedures in concurrently observed groups in the context of randomized studies. Fortanier et al compared anxiety and pain in BM and BC donors. Severe pain from G-CSF, measured on a visual analogue scale, was recorded in 36% of donors compared with 20% in our study. However, they did not observe a difference in pain level between BC and BM donors. The duration of pain was not recorded. A small randomized study by Heldal et al found that the total burden of complaints and analgesic usage was higher in BM donors compared with those giving BC. Finally, Rowley et al reported that BC and BM donors experienced similar peak levels of pain, but that pain lasted longer in BM donors. Fatigue does not appear to have been compared directly. We found that it was a frequent complaint and, in BM donors, is probably attributable in part to anaemia.

This institution's practice of routinely inserting an internal jugular catheter into BC donors is relatively unusual and controversial. Line insertion under imaging seems to be safe; since the closure of this study, more than 200 additional donors have had these catheters inserted, with no major complications. It is noteworthy, however, that the preference for BM donation expressed by three donors was related to discomfort from line insertion. Significant distress can be alleviated in most BC donors with appropriate anaesthesia, sedation, and operator technique. Jugular line access allows the vast majority of collections to be performed in a single leukapheresis, and avoids the additional distress of large vein cannulation if peripheral access fails.

Our study indicates that both procedures are reasonably well tolerated, with all donors recovering within a few weeks. The tolerability of stem cell collection for the donor is but one factor influencing the choice of product. If there is a demonstrable advantage to the patient for one source or the other, the procedure should be selected on this basis. In the absence of such evidence, our data suggest that, at least with respect to pain and fatigue, BC donation is easier to tolerate. No serious consequences attributable to giving G-CSF have been identified in the early years after BC donation; this is consistent with most other reports, although severe effects have been described.

Information from this study is now incorporated into our standard teaching brochure to educate future donors about the potential side effects and expected recovery time with each procedure. This includes a recommendation about the effective contraception in women of childbearing age.

Acknowledgements

We would like to acknowledge the data collection done by Sue Kimber, RN.

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Mini review

The whys and hows of hematopoietic progenitor and stem cell mobilization

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Summary:

Intentional mobilization of hematopoietic/stem cells into the circulation has improved the efficiency of their collection. Transplantation of mobilized blood stem cells to patients with marrow aplasia results in a faster pace of hematopoietic recovery than transplantation of marrow-derived stem cells. Autologous and allogeneic hematopoietic stem cell transplantation are increasingly performed with blood-derived cells. Donors of both autologous and allogeneic blood stem cells do not always respond well to therapies designed to produce mobilization. Autologous donors may respond poorly as a result of myelotoxic damage inflicted by prior antitumor therapy, but this explanation is not valid for allogeneic donors. The mechanism(s) involved in the process of mobilization are incompletely understood. Until these mechanisms are elucidated, methods to improve mobilization vigor on a rational basis will not be obvious. In the meanwhile, clinical observations may provide some hints regarding the whys and hows of mobilization and permit incremental improvements in this process.

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Keywords: mobilization; progenitor; stem cell

In contrast to beliefs persisting into the 1960s that hematopoietic stem cells normally existed only in the bone marrow,¹ the evidence favoring the existence and usefulness of circulating stem/progenitor cells has a long and progressive history (Table 1).³ ¹³

Buoyed by reports that circulating leukemic stem/progenitor cells could be successfully transplanted,² two syngeneic transplants were attempted in 1979 and 1980 with normal blood stem cells.¹⁴ ¹⁵ The cells were collected during steady state and infused over 14 days in the first transplant⁴ and over 8 days in the second.¹⁵ After nearly 2 months with no evidence of engraftment, syngenic marrow transplants from the same donors restored hematopoiesis, resulting in admonitions regarding the clinical use of normal circulating blood stem/progenitor cells for transplantation. Some years later, infusion of autologous cryopreserved blood cells collected during steady state and containing both fewer colony-forming units granulocyte macrophage (CFU-GM) progenitors and mononuclear cells than the failed syngeneic transplants resulted in hematopoietic recovery.¹⁶ Thus, the protracted infusion time of the syngeneic products may have negatively influenced engraftment.

Mobilization of hematopoietic stem/progenitor cells to the circulation

Between 1986 and 1991, autologous peripheral blood stem cell graft products were collected either in steady state or after chemotherapy-induced mobilization (Table 2). As expected, fewer apheresis procedures were needed to collect mobilized vs steady-state stem cells, but mobilized cells provided an unanticipated benefit, faster hematopoietic recovery after transplantation.²⁵ This still incompletely understood phenomenon was responsible for the eventual shift from bone marrow to blood as the preferred source of autologous hematopoietic stem cells for transplantation.

In 1988, two hematopoietic cytokines, granulocyte–macrophage colony-stimulating factor (GM-CSF)²⁶ and granulocyte colony-stimulating factor (G-CSF)²¹ were shown to mobilize hematopoietic stem/progenitor cells to the blood stream. When the cytokines were administered after myelosuppressive chemotherapy, the mobilization effect was even greater.²⁰ Thus, in current clinical practice, chemotherapy is no longer used alone as a mobilizing agent but is combined with a hematopoietic cytokine, most often G-CSF. The mobilizing chemotherapy is either cyclophosphamide in doses of 1.5–7 g/m² or chemotherapy specific for the underlying malignancy. In those situations where administration of chemotherapeutic agent(s) is not appropriate, for example, allogeneic donors and some autologous donors in complete remission, cytokines alone are used to induce mobilization (Table 2).²³

Usefulness of specific mobilization therapies

Chemotherapy plus cytokine(s)

Prospective and retrospective inquiries have shown that chemotherapy, especially when given as part of the specific therapy of the underlying malignancy, followed by a
Table 1  Historical background on circulating stem cells

<table>
<thead>
<tr>
<th>Dates</th>
<th>Observation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1909</td>
<td>Suspicions of circulating stem/progenitor cells</td>
<td>2</td>
</tr>
<tr>
<td>1960s</td>
<td>Stem/progenitor cells identified in circulation of laboratory animals</td>
<td>3,4</td>
</tr>
<tr>
<td>1970s</td>
<td>Circulating stem/progenitor cells identified in man</td>
<td>5,6</td>
</tr>
<tr>
<td>1960–1976</td>
<td>Transplanted circulating cells restore hematopoietic function in lethally</td>
<td>7,10</td>
</tr>
<tr>
<td></td>
<td>irradiated animals</td>
<td></td>
</tr>
<tr>
<td>1974</td>
<td>Large-scale collection of circulating hematopoietic stem/progenitor cells</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>using a blood cell separator</td>
<td></td>
</tr>
<tr>
<td>1977</td>
<td>Recovery of hematopoietic function following autologous transplantation</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>of circulating stem/progenitor cells collected from patients with</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chronic myelogenous leukemia in chronic phase,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>but second chronic phase is short lived.</td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>Successful autotransplantation of blood stem/progenitor cells collected</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>during steady-state hematopoiesis</td>
<td></td>
</tr>
</tbody>
</table>

Table 2  Advances in mobilization of stem/progenitor cells

<table>
<thead>
<tr>
<th>Dates</th>
<th>Mobilization method</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1976–1991</td>
<td>None (steady state)</td>
<td>Multiple apheresis</td>
<td>16</td>
</tr>
<tr>
<td>1976–1991</td>
<td>Chemotherapy</td>
<td>2 to 3-fold increase in progenitor cells 2-3 weeks after chemotherapy</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25-fold increase in CFU-GM in acute leukemia patients after induction</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chemotherapy</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The above cells completely restored hematopoiesis after high-dose therapy</td>
<td>19</td>
</tr>
<tr>
<td>1991–2002</td>
<td>Cytokine + chemotherapy</td>
<td>Cytokines suitable for normal donors; cytokine plus chemotherapy mobilizes more efficiently</td>
<td>20,21</td>
</tr>
</tbody>
</table>

cytokine or cytokine combination yields higher numbers of autologous CD34+ cells than cytokines alone. One randomized trial that supports these observations was designed to limit as much as possible the variables associated with mobilization efficacy. Patients each received two different mobilization regimens, which were alternately administered first or second. Cyclophosphamide plus G-CSF mobilized CD34+ cells more effectively than GM-CSF plus G-CSF regardless of whether that therapy was given as the first or second mobilizing strategy.

Several randomized studies have approached the issues of which cytokine or cytokine combination and at what doses given after chemotherapy provide the best mobilization. A higher (16 g/kg) rather than a lower (8 g/kg) dose of G-CSF was more effective in patients with a variety of malignancies. G-CSF or GM-CSF followed by G-CSF was more effective in patients with solid and hematologic malignancies than GM-CSF. In contrast, for patients with non-Hodgkin’s lymphoma (NHL), GM-CSF followed by G-CSF permitted more efficient collection of a target number of CD34+ cells than did GM-CSF, while G-CSF was the least efficient of the three cytokine strategies. However, cyclophosphamide plus either G-CSF, GM-CSF or GM-CSF plus interleukin 3 (IL-3) mobilized equally well in another trial. Studies including erythropoietin (EPO) have found that it did and that it did not increase mobilization when added to G-CSF after chemotherapy. When added to GM-CSF followed by G-CSF, EPO provided more mobilization than when added to G-CSF. The sometimes conflicting outcomes of these prospective studies (Table 3) are confounded by known and unknown variables in the populations studied (for example, different mobilizing chemotherapy regimens, different underlying diseases, previous treatment with different antitumor agents for different lengths of time) and suggest that a single regimen will not prove optimal for every autologous donor.

**Cytokine(s) alone**
Chemotherapy cannot be added to cytokines for mobilization purposes in normal donors and cytokines alone are
Table 3  Randomized trials of cytokine plus chemotherapy mobilization

<table>
<thead>
<tr>
<th>Cytokine(s)</th>
<th>Chemotherapy</th>
<th>Underlying disease</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>G-CSF 8 μg/kg</td>
<td>Disease specific</td>
<td>Variety of malignancies</td>
<td>28</td>
</tr>
<tr>
<td>G-CSF 16 μg/kg</td>
<td>Disease specific</td>
<td>Variety of malignancies</td>
<td>29</td>
</tr>
<tr>
<td>G-CSF*</td>
<td>Cyclophosphamide</td>
<td>NHL</td>
<td>30</td>
</tr>
<tr>
<td>GM-CSF + G-CSF</td>
<td>Cyclophosphamide</td>
<td>NHL, breast cancer</td>
<td>31</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Disease specific</td>
<td>Ovarian cancer</td>
<td>32</td>
</tr>
<tr>
<td>GM-CSF + IL-3</td>
<td>Disease specific</td>
<td>Breast cancer</td>
<td>33</td>
</tr>
<tr>
<td>GM-CSF + EPO</td>
<td>Disease specific</td>
<td>Ovarian cancer</td>
<td>34</td>
</tr>
<tr>
<td>G-CSF + EPO</td>
<td>Disease specific</td>
<td>Ovarian cancer</td>
<td>34</td>
</tr>
<tr>
<td>EPO + GM-CSF + G-CSF</td>
<td>Disease specific</td>
<td>Ovarian cancer</td>
<td>34</td>
</tr>
</tbody>
</table>

*Statistically higher number of CD34+ cells.

Blood stem cell mobilization
A Nessinger and JG Sharp

employed. G-CSF is the most commonly used cytokine for this purpose. Higher doses of G-CSF and administration of the cytokine twice rather than once daily appear to mobilize more allogeneic CD34+ cells. The combination of G-CSF and GM-CSF did not increase CD34+ cell mobilization in normal donors when retrospectively compared with mobilization with G-CSF alone, but a combination of G-CSF plus GM-CSF was reported to mobilize allogeneic CD34+ cells more effectively than G-CSF alone.

The use of cytokines alone for mobilization in autologous donors with malignancies in remission, who might benefit by avoiding the risks of myelosuppressive chemotherapy holds some attraction. In a retrospective comparison, higher doses of G-CSF (32 μg/kg/day) mobilized more CD34+ cells than lower doses (10 μg/kg/day) in patients with characteristics predicting poor mobilization. Stem cell factor (SCF), a cytokine that acts on primitive hematopoietic stem cells and is available in most countries as an investigational agent, added to G-CSF may also provide a more vigorous mobilization than G-CSF alone. More patients who had been heavily pre-treated for lymphoma collected insufficient cells for transplant after receiving G-CSF (26%) than after receiving G-CSF plus SCF (15%) in a randomized study. Another prospective randomized comparison of G-CSF and G-CSF plus SCF for mobilization in patients with breast cancer showed that those receiving the combination required fewer apheresis procedures to collect progenitor cells for transplant. GM-CSF-mobilizes cells adequately and is an alternative to G-CSF as a single cytokine mobilizer. Other single cytokines have been explored as autologous mobilizers, including EPO, IL-3, a fusion product of GM-CSF and IL-3, FLT3 ligand, and IL-6. They all had modest activity but were not as useful as G-CSF or GM-CSF. These two cytokines, and especially G-CSF, remain the mainstay mobilizing cytokines and no new or novel cytokine seems likely to be adopted for this use in the near future.

Mobilization efficiency and outcome

Approximately 5 x 10^6 transplanted autologous CD34+ cells/kg can be relied upon to provide optimal red and white blood cell recovery, although lower CD34+ doses have performed equally well. The pace of circulating platelet recovery increases with higher doses of CD34+ cells. A total of 15 x 10^6 or more autologous CD34+ cells/kg resulted in a recovery of circulating platelet counts to 50 x 10^3/l in a median of 11 days post transplant as compared with 14 days for patients receiving fewer CD34+ cells but at least 2.5 x 10^6/kg. These patients also maintained platelet counts of >20 x 10^9/l beginning a median of 10 days post transplant compared to a median of 8 days for those receiving the higher doses. Unless patients are bleeding, platelet transfusions are not usually used for counts >20 x 10^9/l. The benefits of recovery of circulating platelet counts earlier than 10 days after transplant may not be outweighed by the increased resources required to collect more than 2.5-5 x 10^9 autologous CD34+ cells/kg.

Whether identifying the most efficient mobilization therapy among the several demonstrated effective strategies is possible, important or even necessary is an intriguing question. If one strategy routinely produces sufficient number of cells in a single apheresis procedure to assure rapid and reliable hematopoietic recovery, is another strategy that provides twice as many cells preferable? The answer would be yes if infusion of higher doses of circulating mobilized stem/progenitor cells provide added benefit beyond rapid durable engraftment without added toxicity.

Some indirect evidence for added benefits of transplantation of higher numbers of allogeneic stem/progenitor cells
than needed for rapid hematopoietic recovery came from a randomized prospective study comparing mobilized blood-derived stem cell vs marrow-derived stem cell transplants. Patients transplanted with blood stem cells had better outcomes as regards disease-free survival and overall survival.\(^\text{51}\) Whether the improvement was related to the larger number of stem/progenitor cells in a blood stem cell vs a marrow stem cell graft product\(^\text{52}\) was neither discounted nor demonstrated. A second smaller randomized study of allogeneic marrow vs mobilized blood stem cell transplantation found that patients who received fewer than \(2 \times 10^6\)/kg CD34+ cells, regardless of the cell source, experienced higher mortality and poorer survival.\(^\text{53}\) Another potential benefit of transplantation of higher stem cell doses in the allogeneic setting is a more complete recovery of circulating lymphocytes.\(^\text{54}\) In the autologous arena, a study of bone marrow transplantation in CML patients revealed that higher numbers of CD34+ cells infused was associated with a decreased risk of death and decreased +100 day transplant-related mortality. Whether this observation translates to the blood stem/progenitor cell arena is unknown.\(^\text{55}\) Thus, clinical benefits to very high doses of CD34+ cells for transplantation in the autologous setting may exist but none have been positively identified. As regards any additional toxicities associated with transplantation of more cells than required for rapid hematopoietic recovery, increasing allogeneic CD34+ cell doses correlated with a higher likelihood of clinical extensive chronic graft versus host disease, suggesting that increasing the CD34 cell numbers in mobilized graft products may be counterproductive.\(^\text{56}\) Transplanted allogeneic CD34+ positively selected cell doses of \(1-3 \times 10^6\)/kg have been associated with increased survival compared with higher doses.\(^\text{57}\) Taken together, these few studies do not establish whether higher doses of hematopoietic stem/progenitor cells are of any additional advantage but may provide a disadvantage in the allogeneic setting. Consequently, the optimal number of infused blood-derived stem/progenitor cells remains uncertain.

Mobilization of malignant cells

In addition to mobilizing hematopoietic stem/progenitor cells, cytokines\(^\text{58}\) and chemotherapy plus cytokines\(^\text{59}\) have also mobilized malignant cells in the blood stream, although tumor cell mobilization has not been identified in every studied autologous collection.\(^\text{60,61}\) Autologous hematopoietic stem cell donors with follicular NHL were more likely to have detectable tumor cells in their apheresis products if they mobilized CD34+ cells poorly (42%) rather than well (17%), regardless of the mobilizing therapy used.\(^\text{62}\) One study found that poor mobilizers were more likely to experience lymphoma relapse post-transplant, but the infused products were not assayed for tumor cells.\(^\text{63}\) Other investigators, however, found no differences in event-free survival, overall survival or relapse when the outcomes of good mobilizers and poor mobilizers with NHL treated with high-dose therapy and transplantation were compared.\(^\text{64}\) These results support an earlier finding that patients with low-grade NHL who received either mobilized or steady-state blood stem/progenitor cells following high-dose therapy had similar event free and overall survival.\(^\text{65}\) While avoiding increased tumor cell contamination in autologous mobilized blood stem cell collections seems intuitively desirable, no evidence exists to suggest that infusion of mobilized (in contrast to nonmobilized\(^\text{11}\)) tumor cells in the graft product increases the incidence of relapse of NHL,\(^\text{66}\) multiple myeloma\(^\text{67}\) or breast cancer\(^\text{68}\) following transplant.

Poor response to mobilization therapy

Autologous donors

The response of individual donors to mobilizing therapies is variable and incompletely predictable.\(^\text{69}\) A total of 21-48% of patients with NHL have exhibited poor mobilization after administration of chemotherapy and G-CSF.\(^\text{63,64,69}\) Of these patients, 16-33% were unable to mobilize enough collectable stem/progenitor cells for transplantation.\(^\text{63,69}\) Factors predicting for a higher likelihood of poor mobilization include increasing numbers of cycles of prior antitumor chemotherapy administration,\(^\text{70}\) prior radiation therapy,\(^\text{70,71}\) follicular rather than diffuse NHL as the underlying disease,\(^\text{70}\) and the presence of overt marrow metastases.\(^\text{25}\) In donors with lymphoma, low numbers of circulating natural killer (NK) CD3-16+56+ cells prior to administration of mobilizing therapies have predicted poor mobilization,\(^\text{72}\) as have low platelet counts on the first day of autologous blood stem/progenitor cell collection.\(^\text{73}\) Older age was associated with poor autologous mobilization in some patient groups,\(^\text{80,74,75}\) but not in others.\(^\text{74,76}\) Even though some donors with predictors of poor mobilization respond quite well to mobilizing therapies,\(^\text{77}\) poorly mobilizing autologous donors are assumed to have sustained an injury to the hematopoietic stem cell system that is responsible for the poor effect (see Table 4). In support of this premise, autologous donors with breast cancer who had received more chemotherapy and radiation or had tumor metastases in the marrow also had fewer stem/progenitor cells in apheresis products collected during steady state than donors who were less heavily pretreated and had no marrow metastases.\(^\text{78}\) Such damage may not account for all of the poor mobilization encountered in autologous donors.

### Table 4 Factors associated with poor mobilization

<table>
<thead>
<tr>
<th>Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>More cycles of prior myeloablative chemotherapy</td>
<td>70</td>
</tr>
<tr>
<td>Prior radiation therapy</td>
<td>70,71</td>
</tr>
<tr>
<td>Follicular rather than diffuse NHL</td>
<td>70</td>
</tr>
<tr>
<td>Overt marrow metastases</td>
<td>25</td>
</tr>
<tr>
<td>Low circulating NK cell numbers</td>
<td>72</td>
</tr>
<tr>
<td>Low platelet counts</td>
<td>73</td>
</tr>
<tr>
<td>Older age (in some studies, not in others)</td>
<td>30,74,75,76</td>
</tr>
<tr>
<td>Prior rituximab therapy</td>
<td>79</td>
</tr>
</tbody>
</table>
example, patients who had received rituximab within 6 months of mobilization therapy mobilized less vigorously than rituximab-naïve patients. The use of rituximab during mobilization therapy for in vivo purging has not negatively affected mobilization.

**Allogeneic donors**

In the mid-1990s, normal donors with no history of cancer therapy began to receive mobilizing cytokines as allogeneic peripheral blood stem cell transplantation became more commonplace. Of these donors, 4-20% were noted to be poor mobilizers. The varying percentages are likely due, in part, to differences in the definition of poor mobilization. Nonetheless, these observations in normal donors established that a stem cell pool compromised by prior therapy, metastases or aging is not the only explanation for poor mobilization.

The causes of poor allogeneic stem cell mobilization are elusive, but some hints have recently come to light. DBA-strain mice exhibit rapid and vigorous mobilization when treated with G-CSF, Balb/c mice demonstrate delayed but vigorous mobilization and C57Bl/6 mice mobilize poorly. When plasma from C57Bl/6 mice was injected into Balb/c mice just prior to administration of mobilizing cytokines, mobilization was inhibited. Plasma from Balb/c and DBA mice was less inhibitory, suggesting that a genetically controlled circulating factor in the blood could be responsible for regulating the vigor and timing of mobilization.

A mouse model of poor mobilization was constructed by exposing Balb/c mice to lower-half-body irradiation prior to administration of mobilizing cytokines. These mice did not exhibit mobilization following cytokine administration. When plasma from part-body irradiated mice was injected into intact mice before cytokine administration, no mobilization resulted, suggesting that a circulating factor was responsible for the inhibition. Injection of plasma from poorly mobilizing human donors into mice prior to administration of mobilizing cytokine also inhibited mobilization. Whether this circulating factor(s) is operative in the clinical situation of poor mobilization in allogeneic and autologous donors is unknown. Since the survival of poorly mobilizing autologous patients with NHL was inferior to that of rapid mobilizers post-transplant and tumor relapse was more likely for poor mobilizers, identification and inactivation of any circulating factor that inhibits mobilization could improve patient outcome (Table 2).

**Molecular markers of mobilization**

Methods to improve the vigor of mobilization are virtually impossible to design on a rational basis since the mechanisms involved in the process are largely unknown. Stem cells reside in marrow adherent to marrow stromal cells via αβ integrin-mediated adhesion, that is, very late antigen 4 (VLA-4) on the stem cell and vascular cell adhesion molecule-1 (VCAM) on the stromal cell. Interruption of adhesion releases stem cells to the circulation. A higher percentage of CD34+ cells expressing VLA-4 was observed among the mobilized stem/progenitor cells of good mobilizers with NHL than in those of poor mobilizers. Marrow stromal cells produce a chemokine, stromal-derived factor 1 (SDF1), whose receptor, CXCR4, is present on CD34+ cells. Patients with NHL treated with chemotherapy plus cytokine(s) who mobilized well had significantly lower plasma levels of SDF-1 and lower percentages of CD34+ cells in the collections expressing CXCR4 than poor mobilizers. High plasma levels of flt3-ligand prior to administration of mobilizing therapies predict poor mobilization of autologous, but not allogeneic CD 34+ cells. Prospective studies are needed to confirm that molecular markers can accurately identify poor autologous mobilizers prior to administration of mobilizing therapies (Table 5).

**Harvesting enough cells for transplant from poor mobilizers**

Reports of maneuvers to harvest more stem/progenitor cells from donors who responded poorly to initial mobilizing therapies will be reviewed, but translation of the results to clinical practice is confounded by multiple variables. For example, a graft product anticipated to provide rapid, complete and durable restoration of marrow function at one center could be considered unacceptable at another. Failure to reach targets between 1 x 10^6/CD34+ cells/kg and 3 x 10^6 CD34+ cells/kg has defined products unsuitable for transplant at individual institutions. Methods to detect poor mobilization also vary and include sampling peripheral blood rather than determining the

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Key areas requiring additional investigation</th>
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</thead>
<tbody>
<tr>
<td><strong>Area</strong></td>
<td><strong>Challenges</strong></td>
</tr>
<tr>
<td>Accurate prospective identification of poor mobilizers</td>
<td>Current clinical identification, imprecise</td>
</tr>
<tr>
<td>Defining optimal numbers of infused stem/progenitor cells</td>
<td>Increased allogeneic numbers provide more chronic GVHD and faster lymphocyte recovery</td>
</tr>
<tr>
<td>Identification and inactivation of mobilization inhibitors</td>
<td>Stem cell damage is not the only cause of poor mobilization</td>
</tr>
<tr>
<td>Definition of mechanisms of mobilization</td>
<td>Required to develop logical approach to improve mobilization</td>
</tr>
</tbody>
</table>

Bone Marrow Transplantation
number of cell population subsets such as CD34+ cells and/or CFU-GM in an apheresis product. Some centers perform several apheresis procedures if needed to procure the target numbers of cells, while others do not. The volume of blood processed by the blood cell separator for each collection has varied from 310 to 351 and more. Keeping these differences in mind, maneuvers reported to bolster collection numbers for donors who mobilized poorly following various mobilization therapies are discussed.

**Poor autologous mobilization response to myelosuppressive chemotherapy**

Chemotherapy alone was used for mobilization before hematopoietic cytokines were available. Remobilization with higher cyclophosphamide doses in a small group of poor mobilizers with a variety of malignancies provided increased numbers of CFU-GM in the apheresis products while remobilization with the same dose of cyclophosphamide did not. Although chemotherapy without cytokines is no longer used for mobilization purposes, the observation that increasing doses of chemotherapy correlated with increasing vigor of mobilization could be useful when designing remobilization strategies.

**Poor autologous mobilization response to myelosuppressive chemotherapy plus cytokines**

Autologous donors with lymphoma, myeloma and Ewing's sarcoma who were treated with chemotherapy and G-CSF, and did not attain a suitable graft product with three apheresis procedures were remobilized with essentially the same mobilizing therapies. More CD34+ cells and CFU-GMs were collected during the second mobilization than the first from most donors. When collections from the first and second mobilizations were combined, 70% of the patients had sufficient cells for transplant. In another instance, a group of autologous donors with a variety of malignancies who mobilized poorly after treatment with disease-specific chemotherapy plus 5 μg/kg G-CSF were remobilized with either the same therapy or with 10 μg/kg G-CSF alone. Those receiving G-CSF alone yielded significantly higher numbers of CD34+ cells and CFU-GM than during their initial mobilization, while those remobilized with the same therapy yielded similar cell numbers to those with the first mobilization. However, the numbers of cells collected during either of the remobilization strategies were approximately the same. The value of cytokines alone as remobilizing therapy was also studied in another group of 23 autologous donors with solid tumors and hematologic malignancies who collected insufficient cells for transplant after up to five apheresis procedures. Initial mobilization had been accomplished with chemotherapy, G-CSF or a combination of chemotherapy and G-CSF. Remobilization therapy was initiated within 35 days using a combination of G-CSF and GM-CSF. The median number of CD34+ cells collected during remobilization was significantly higher than during the initial mobilization, although four of the 23 donors studied had poorer collections with remobilization. The largest study of remobilization in poorly mobilizing autologous donors described 119 patients with a good performance status whose initial mobilization therapy was either G-CSF and chemotherapy or G-CSF alone. The initial collections contained less than 2.5 x 10^6 CD34+ cells/kg. Remobilization was accomplished with either chemotherapy and G-CSF or G-CSF alone. Significantly, more CD34+ cells were collected during the second mobilization attempt than during the first, regardless of which remobilization therapy was used; G-CSF alone was as effective as chemotherapy plus G-CSF in remobilizing CD34+ cells. Thus, for patients who mobilized poorly with chemotherapy and cytokine, remobilization using the same therapy again, using cytokine alone at higher doses, or a combination of cytokines alone when a single cytokine was originally combined with chemotherapy, was successful in most but not in all patients.

**Poor autologous mobilization response to cytokines**

Studies specifically addressing the question of remobilization in autologous donors who mobilized poorly following cytokine administration are sparse. Since chemotherapy plus cytokine has been shown to mobilize more effectively than cytokine alone, this strategy might be tried for remobilization, provided neutropenia is an acceptable risk for the patient. Since good mobilizers have responded better to higher doses of cytokines than lower doses, and donors who mobilized poorly after chemotherapy plus cytokines have responded better to higher doses of cytokine or cytokine combinations, these strategies could be viable options for remobilization. For those few patients with obvious disease progression who mobilize poorly with cytokines alone, remobilization with disease-specific chemotherapy plus cytokines has often resulted in a better mobilization response in the experience of the authors.

**Autologous bone marrow harvests from poor mobilizers**

Marrow has occasionally been harvested from poorly mobilizing autologous donors in an attempt to construct a suitable graft product. A total of 13 patients with a variety of malignancies failed to mobilize target numbers of CD34+ cells prior to initiating apheresis and underwent marrow harvesting rather than blood stem cell collection. Nine were transplanted with the marrow after high-dose therapy and experienced delayed platelet recovery compared to a group of autologous marrow transplant patients that served as a historical control. Whether a suitable graft product could have been collected from the peripheral blood of those patients is unknown, but the marrow-derived graft product was inferior. Autologous bone marrow harvests with good cellularity but unknown numbers of CD34+ cells were harvested from another group of patients who had mobilized poorly. The marrow cells were added to the blood-derived progenitor cells and transplanted to 11 evaluable patients. Delayed hematopoietic recovery and a high (42%) procedure-related mortality resulted, leading to the conclusion that marrow harvested from poor mobilizers is an unreliable product. The number of publications regarding marrow harvests in
poorly mobilizing donors is small, but available data suggest that the transplantation quality of the marrow is poor.

**Poor allogeneic mobilization response to cytokines**

Little information is available regarding strategies to improve the vigor of mobilization in poorly mobilizing allogeneic donors. However, remobilization in good mobilizers to obtain a second allogeneic graft product has been described. In a single case report, retreatment with a mobilizing cytokine 3 days after completing a successful apheresis collection resulted in a poor CD34+ cell yield, suggesting that a longer time period is needed before retreatment with mobilizing cytokines. This observation was confirmed in a report of 13 allogeneic donors who underwent remobilization at a median of 3 months (range 1–13 months) after the initial mobilization and the number of CD34+ cells harvested during the first and second mobilization were comparable. Another group of 10 donors were mobilized twice with G-CSF for allogeneic blood stem/progenitor cell collection. The median time between the first and second mobilization was 41.5 days (range 16–385 days). Shorter time intervals between the two mobilization events were associated with fewer CD34+ cells collected during the second mobilization period. However, after 60 days, the CD34+ cell content of the first and second collections were similar. The only patient who received twice as much cytokine during the second mobilization than the first had more CD34+ cells in the second collection. Taken together, these reports suggest that if a poorly mobilizing allogeneic donor is considered for remobilization, a time interval of a month or two should be considered between the two collections if possible. If a month’s wait is not feasible, then remobilization with a higher dose of growth factor might be helpful.

**Transplantation of cells from poorly mobilizing donors**

A minimum of $1 \times 10^6$ CD34+ cells/kg is considered essential to assure hematopoietic recovery following transplant, with higher doses providing more rapid platelet recovery. A total of 18 patients who collected $< 1 \times 10^6$ kg mobilized CD34+ cells/kg and $< 1 \times 10^4$ CFU-GM were transplanted with those cells after high-dose therapy. Six patients received only those cells and five experienced delayed engraftment. A cellular marrow harvest was added to the cells for transplant to the remaining 12 patients. Of 11 evaluable patients, four had delayed neutrophil and eight delayed platelet engraftment, suggesting suitable graft products cannot be reliably constructed by adding marrow harvests to inadequate numbers of poorly mobilized blood stem/progenitor cells.

Three reports regarding transfusion of re-mobilized cells are instructive. A group of 23 patients whose mobilized collections contained fewer than $3 \times 10^6$ CD34+ cells/kg were remobilized with G-CSF and GM-CSF. Cells collected during both mobilization attempts were combined and transplanted following high-dose therapy. In all, 20 patients received at least $2 \times 10^6$ CD34+ cells/kg and recovered neutrophils at a median of at least 13 days and platelets at a median of at least 27 days. A second report of transplantation using remobilized cells described 27 patients who collected less than $2 \times 10^6$ CD34+ cells/kg and/or less than $10 \times 10^4$ CFU-GM/kg during the first mobilization. Both cell collections were combined and 24 received high-dose therapy and transplantation of $1.03-5.85 \times 10^6$ CD34+ cells. All but one patient recovered hematopoietic function by a median of 26 days (platelets, reticulocytes and neutrophils) after infusion. A third group of 49 patients who failed to collect $1 \times 10^6$ CD34+ cells after the first mobilization was remobilized and the collected cells were combined. The patients were treated with high-dose therapy and $0.88-6.74 \times 10^6$ autologous CD34+ cells/kg were infused. Recovery of $0.5 \times 10^9$/granulocytes to the circulation occurred at a median of 11 (9-16) days for 48 evaluable patients and platelet recovery occurred at a median of 14 (6-85) days for 47 evaluable patients. These three reports suggest that, with remobilization, enough hematopoietic stem/progenitor cells can be collected from most poorly mobilizing autologous donors to construct a transplant product that provides timely hematopoietic recovery.

**A mobilization strategy**

Taken together, these studies can support more than one evidence-based algorithm for mobilization of hematopoietic progenitor/stem cells. One general approach is to identify donors at high risk for poor mobilization and provide them with therapies aimed to provide maximum mobilization vigor, accepting any increased time, cost, resource utilization and/or donor risk for morbidity that might be associated with the selected therapy. A second general approach is also supportable, recognizing that while groups of autologous donors can be categorized as being at high risk for poor mobilization, individual autologous and allogeneic donors who will mobilize poorly cannot be accurately identified prospectively. Since most donors are good or adequate mobilizers, all donors are treated with therapies known to produce useful mobilization in most cases, saving the more rigorous and/or costly therapies for those who respond poorly.

Cytokine administration produces sufficient mobilization to harvest $1.5-4 \times 10^6$ CD34+ cells/kg in one to four apheresis procedures for most donors at the University of Nebraska Medical Center. The entire collection process from the time of administration of the first dose of cytokine until the final apheresis procedure is complete in 5–9 days when cytokine administration begins on Thursday and the first apheresis procedure begins the following Monday. If chemotherapy is given specifically for mobilization, the process will require 14–16 days from the date of chemotherapy administration until the graft product is collected. If chemotherapy given as part of disease-specific therapy is also used for mobilization, the time saving is optimal. Remobilization is performed for those patients who do not amass a sufficient number of cells for transplant during the first mobilization attempt (Table 6).
Table 6  Algorithms of mobilization strategies

<table>
<thead>
<tr>
<th>Autologous donor</th>
<th>Allogene donor</th>
</tr>
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<tbody>
<tr>
<td>For patients with malignancy in remission</td>
<td>10 µg/kg G-CSF/day, begin apheresis on 5th day</td>
</tr>
<tr>
<td>10 µg/kg G-CSF/day, begin apheresis on 5th day</td>
<td></td>
</tr>
<tr>
<td>For patients receiving standard-dose chemotherapy to achieve remission</td>
<td>Disease-specific chemotherapy followed by G-CSF. Begin apheresis when WBC begins to recover</td>
</tr>
<tr>
<td>For patients who mobilize poorly after the above therapies</td>
<td>If previously mobilized with cytokine only</td>
</tr>
<tr>
<td>Remobilize with chemotherapy plus cytokine, if appropriate or remobilize with same or higher doses of cytokine</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion

Cytokines alone or chemotherapy plus cytokines mobilize hematopoietic stem cells to the circulation. Mobilized hematopoietic stem/progenitor cells provide rapid hematopoietic recovery following transplantation. While most donors mobilize well, a percentage of both allogeneic and autologous donors respond poorly to mobilizing therapies. A single solution to poor mobilization is unlikely to exist, since the reasons for the problem are multiple and differ from one donor to the next. Until the causes are accurately defined, solutions are unlikely to be found. The issue is important not only for current transplant needs, but also for future needs if, as early reports suggest, these circulating hematopoietic cells are capable of transdifferentiation and will be used to engineer nonhematopoietic cells and tissues. Hopefully, additional research will optimize mobilization to the extent that a sufficient number of cells for any need can be harvested with a simple phlebotomy.

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special communication

JACIE Accreditation in 2008: demonstrating excellence in stem cell transplantation

Penwood Pamphil, Jane F. Apperley, Diana Samson, Ineke Slaper-Cortenbach, Eoin McGrath

JACIE was initiated as a small pilot project in Spain in 2000 and launched as a formal Europe-wide inspection program in January 2004. Since 2000, over 150 applications for accreditation have been received by the JACIE Office and more than 130 inspections have been completed in European centers and facilities. Almost all of these were found to be functioning at a high level of excellence, with the majority having only minor deficiencies in compliance with the standards. In one-third of centers there were more significant deficiencies. The most common deficiencies were in quality management. Following correction of deficiencies 86 centers have to date achieved full accreditation and many more are nearing the completion of the process. Implementation of JACIE involves a significant investment of time and resources by applicant centers. The majority require at least 18 months to prepare for accreditation and 85% have needed to employ a quality manager and/or data manager on an ongoing basis. However, all centers felt their program had benefited from the implementation of JACIE. JACIE is also working closely with other international organisations related to cellular therapy as part of the Alliance for the Harmonisation of Cell Therapy Accreditation (AHCTA), which is examining the differences in existing standards and aiming to develop international standards for all aspects of stem cell transplantation. In particular the requirements for safety of imported tissues and cells has emphasised the need for global harmonisation. The recent implementation of Directive 2004/23/EC and the associated Commission Directives 2006/17/EC and 2006/86/EC has provided an impetus for the implementation of JACIE in European Union (EU) member states. It will be important in the future to examine how JACIE can co-operate with the EU Competent Authorities (CA) to ease the burden of the inspection process for haematopoietic stem cell (HSC) transplant programs.

The Joint Accreditation Committee of ISCT-EBMT (JACIE) is a non-profit body first established in 1998 for the assessment and accreditation of hematopoietic stem cell (HSC) transplantation programs. The Committee was founded by the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Cellular Therapy (ISCT), the two leading scientific organizations involved with HSC transplantation in Europe. JACIE modelled itself on the US-based Foundation for the Accreditation of Cellular Therapy (FACT), established in 1996 by the ISCT and the American Society for Blood and Marrow Transplantation (ASBMT). JACIE works closely with FACT through a joint committee structure to establish standards for the provision of quality medical and laboratory practice in HSC transplantation. JACIE conducts inspections, accredits transplant programs and encourages health institutions and facilities performing HSC transplantation to voluntarily meet these standards to demonstrate their high levels of quality of care.

The primary aim of JACIE is to improve the quality of HSC transplantation in Europe by providing a means whereby clinical transplant centers, HSC collection facilities and processing facilities can demonstrate excellence. This is supported by its co-ordinating role in the provision of training courses in quality management for applicant centers and training courses for inspectors. An additional and wider aim is to ensure harmonisation between JACIE standards and other national/international standards, including the EU Tissues & Cells Directive 2004/23/EC and the related implement-
JACIE accreditation is voluntary, but provides a means whereby transplant facilities can demonstrate that they are working within a quality system covering all aspects of the transplantation process and thus show compliance with the requirements of insurance companies or national and/or international regulatory authorities.

The JACIE program was run as a pilot program in Spain between 2000 and 2002 and formally launched on an international basis in January 2003 with support from the European Union under the Public Health Program (2003-2008) (http://ec.europa.eu/health/ph_projects/2003/action2/action2_2003_05_en.htm). Between January 2000 and November 2008, 139 centers or facilities have been inspected for the first time and 16 have been re-inspected. The number of applications/inspections per country is Austria 2/3, Belgium 8/3, Czech Republic 3/1, Finland 3/3, France 22/20, Germany 27/20, Italy 17/11, The Netherlands 20/16, Poland 1/0, Saudi Arabia 1/1, Spain 10/7, Switzerland 10/16, Turkey 1/1, and the United Kingdom 33/37 (Figure 1 shows initial applications by country). This experience has enabled JACIE to identify areas of common difficulty for applicant centers, to assess what assurance centers need in order to achieve accreditation, and has also raised some general issues relating to national and international regulation.

The FACT-JACIE Standards

The 4th edition of the joint FACT-JACIE standards covers all aspects of clinical transplant programs, collection facilities (bone marrow [BM] and peripheral blood progenitor cell [PBPC] collection) and processing of HPC, as shown in Table 1. These superseded the 3rd edition at the start of November 2008. The 4th edition will be the standard against which programs are inspected from 1st February 2009. The updated standards are applicable to cellular therapy products (CTP) but where applicable only to hematopoietic progenitor cells (HPC), this is specifically referenced. The standards also apply to the use of therapeutic cells (TC) derived from blood or marrow, including donor lymphocytes. Important changes in the 4th edition are as follows:

1. Quality Management (QM): the standards have been realigned to reduce redundant references to QM topics and have been organized in each section on a topical basis. Standards pertaining to operational quality controls have been relocated to the relevant operations sections
2. Standard Operating Procedure (SOP) review: the requirement has been changed from annual to biannual (or upon the introduction of changes in procedures, whichever is sooner).
### Table 1. Analysis of Most Common Deficiencies

<table>
<thead>
<tr>
<th>Clinical program</th>
<th>Collection facility</th>
<th>Processing facility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Missing or inconsistent donor information e.g. vaccinations, travel, pregnancies and blood transfusion histories</td>
<td>- No OIM plan</td>
<td>- Lack of integration with the facilities</td>
</tr>
<tr>
<td>- Lack of written information e.g. on collection procedures</td>
<td>- Present but with significant omissions e.g. lack of validation procedures</td>
<td>- No validation or qualification studies</td>
</tr>
<tr>
<td>- IDMs</td>
<td>Policies and procedures</td>
<td>Policies and procedures</td>
</tr>
<tr>
<td>- Medical history doesn't include the correct questions</td>
<td>- Present but inadequate</td>
<td>- Present but inadequate</td>
</tr>
<tr>
<td>- Specific tests omitted</td>
<td>- No range of expected outcomes/results</td>
<td>- No range of expected outcomes/results</td>
</tr>
<tr>
<td>- Tests not done within 30 days of HPC transplant</td>
<td>- No procedures for recording deviation</td>
<td>- No procedures for recording deviations</td>
</tr>
<tr>
<td>- Data management</td>
<td>Review of new/revised documents</td>
<td>No reference section</td>
</tr>
<tr>
<td>- Incomplete or incorrect forms</td>
<td>- Failure to undertake or document review</td>
<td>- No reference section</td>
</tr>
<tr>
<td>- Lack of engravement data</td>
<td>- No written request for processing</td>
<td>- Process control</td>
</tr>
<tr>
<td>- Clinical status at HPC transplant not recorded</td>
<td>- No review of processing records</td>
<td>- ABO, Rh tests not done</td>
</tr>
<tr>
<td>- Chemi: lack of prescription</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adverse events, errors and clinical incidents</strong></td>
<td>Collection procedures</td>
<td>Labelling</td>
</tr>
<tr>
<td>- Not reported or recorded</td>
<td>- Insufficient number of BM procedures</td>
<td>- Incorrect product name used</td>
</tr>
<tr>
<td>- Lack of regular audit</td>
<td>- Lack of written order for collection</td>
<td>- Information missing from labels e.g. date/time allocated, volume etc.</td>
</tr>
<tr>
<td>- No corrective actions</td>
<td>- No interim donor checks</td>
<td>- No unique alphanumeric identifier</td>
</tr>
<tr>
<td><strong>Outpatient facilities</strong></td>
<td>Engravement data</td>
<td>Engravement data</td>
</tr>
<tr>
<td>- Lack of space</td>
<td>- Failure to document and review time to engravement</td>
<td>- Failure to document and review time to engravement</td>
</tr>
<tr>
<td>- Inadequate separation of patients with significant infections</td>
<td></td>
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</tr>
</tbody>
</table>

**IDMs** = Infectious disease markers e.g. HIV / HCV

3. The donor selection, evaluation, and management sections in the clinical program and collection facility sections have been modified to reflect the way that these responsibilities are divided between clinical programs and collection facilities. The collection facility standards focus more on donor evaluation and management, with less emphasis on donor selection activities. In some situations the collection facility is primarily responsible for donor selection activities, and the standards now state that in these situations, collection facilities are required to comply with the applicable clinical program standards.

4. Early discharge from the transplant center. It was agreed by the FACT-JACIE Standards Committee that it is against the spirit of the standards to inspect and accredit the center performing the transplant procedure as the "transplant center" without considering post-transplant care.

With this in mind the 4th edition of the standards states that Clinical Programs shall ensure planned discharges are to facilities adequate for post-transplant care. This means that it is the responsibility of the transplant center to ensure compliance with items such as the provision of isolation facilities, staffing and training and policies and procedures. JACIE will require documentation of compliance and this might in the future include inspection of the hospital providing post-transplant care.

5. Appendices have been revised to clarify requirements and simplify the standards. Those that are external tables and forms have been removed and replaced with a reference table indicating the websites where the current versions can be found. This is in response to situations in which external tables and forms are updated within months of publication of FACT-JACIE standards, which causes the appendices to become out of date be-
special communication

Figure 3. Cumulative numbers of initial applications and total inspections between 2000-2008.

Figure 4. Initial applications for inspection by year between 2000-2008.

before the next edition is published.

6. Expanded requirements: an effort has been made to simplify the standards since they are intended to define minimum standards rather than best practice. Some requirements have been expanded and specific examples include:

- Written agreements - the responsibility of ensuring external entities comply with standards and governmental laws and regulations is placed on the clinical program/collection facility/processing facility as appropriate.
- Disaster plans - explicitly states in standard

the requirement to include the response of the clinical program/collection facility/processing facility as appropriate.

- Concurrent plasma and samples are required to have the same identifier as the cellular therapy product.

7. Terminology: in several cases the use of specific terminology was clarified to reduce misinterpretations commonly found during the inspection and accreditation process and to account for international variations. Specific examples include:

- Change from HPC to CTP where applicable. In instances where only HPC applies it is specifically referenced.
- Board eligibility/certification references changed to specialist certification to accommodate international education.
- References to specific governmental agencies and accrediting bodies, e.g. the United States Food and Drug Administration (FDA) etc have been changed to appropriate governmental authorities or certified as required by governmental authorities as appropriate.
- Validation has been redefined to include only processes (including intended uses of equipment) and qualification to include only equipment, supplies, and reagents (in alignment with FDA interpretation).

There are also a number of specific sectional changes which are not described here.

The complete standards and the accompanying guidance manual are available on the FACT and JACIE websites. They are broadly consistent with the requirements of the Tissues and Cells Directive (2004/23/EC) and accompanying Commission Directives (see above) as regards donation, procurement and processing of stem cells, but in addition, cover the clinical transplant program. Accreditation of clinical programs includes the clinical use of cord blood (CB) stem cells but the JACIE program does not accredit CB collection and banking facilities as this process is currently carried out by FACT against the Netcord-FACT Standards.

The FACT-JACIE Manual and Inspection Checklist

The manual contains the standards together with detailed guidance on the interpretation and measures required to demonstrate compliance. Each standard is followed by specific questions relating to that standard and these questions form the basis of the inspection checklist, which must be completed prior to inspection by the applicant center and verified by the inspector during the
inspection. The checklist has recently changed format from Microsoft Word to Microsoft Excel to facilitate the completion of the checklist by both the applicant and the inspector and to make analysis of the answers easier by using automatic colour-coding and including filters (Figure 2).

The Accreditation Process

Preparation by Center
The center implements measures as described in the FACT-JACIE accreditation manual, and then applies for inspection by submitting basic information about the program/facility and a number of supporting documents including a self-assessment checklist. The application information and checklist must be submitted in English but all other documentation, including SOPs is accepted in the language of the center.

Inspection
An on-site visit is carried out by a team of trained inspectors, usually one per facility (clinical / collection / processing). Inspectors are medical, scientific or other professional persons working in HSC transplantation, with specific qualifications and experience for inspecting clinical, collection and / or processing facilities. Inspectors must attend a JACIE-sponsored training course and pass an examination. Where a clinical program performs adult and paediatric transplants, an adult and a paediatric inspector will attend. Inspectors may also be from another country but should be either native or fluent speakers of the relevant language. An inspection visit usually lasts 1.5 days and involves discussion with staff during their work, review of documents / records and completion of a detailed checklist relating to the standards.

The report is prepared in English, notes any areas of non-compliance with the standards and is reviewed by the JACIE Office and Accreditation Committee, the latter established in 2006. This report indicates all non-compliances with specific standards and makes specific recommendations for corrections and improvements. The distinction between minor deficiencies and more significant deficiencies is not strictly defined, but in general terms, specific deficiencies in documentation are considered minor while more general problems with documentation or problems with processes or facilities are considered significant. The center is allowed up to 9 months to correct deficiencies, depending on the amount of work required.

Center Response
The center must indicate acceptance of the findings and then in due course submit documentary evidence to confirm corrections or amendments. The original inspectors review the documentation. Review by the inspectors rather than by the Accreditation Committee is required because of language issues. In some cases a full or limited re-inspection may be required to show that deficiencies have been corrected. The inspectors confirm to the JACIE Office that all necessary corrections have been made or indicate that there are still outstanding areas for completion.

Accreditation
The JACIE Office and Accreditation Committee reviews all the reports and relevant documentation and if satisfied that all deficiencies have been corrected, makes a recommendation to the JACIE Board that the center be awarded accreditation. If approved, accreditation is awarded, valid for 4 years, subject to an annual report from the center noting any significant changes in personnel or processes and including annual activity figures and an interim audit at the end of the second year of accreditation.

Summary of JACIE Inspection Activity
To date, 156 facilities have formally applied for accreditation. Ninety-nine centers applied for accreditation for a combination of clinical, collection and processing facilities; 20 centers applied for clinical only; 1 for bone marrow harvest only; 4 for apheresis collection only; 2 centers for collection and processing only; and 12 for processing only. Between January 2000 and November 2008 139 centers were inspected (Figure 3) including

Figure 5. Provisional data showing the proportion of centers reporting transplants to the 2007 EBMT Activity Survey that have applied for JACIE accreditation and meet the minimum transplant requirements.
both first-time and reaccreditation inspections. The number of first-time or initial applications rose to 33 in 2007 and 31 applications have been received to date in 2008 (Figure 4) although the overall number of applications is higher as a result of requests for reaccreditation. Figure 5 shows the overall proportion of transplant centers reporting to the EBMT that are now JACIE registered.

Common Deficiencies
The most common deficiencies were in documentation, labelling and in the quality management program. This is consistent with the initial experience of the FACT accreditation program in the United States and is described in more detail in the next section. Minor failures of compliance were frequent, usually involving problems with documentation. Examples include:
- SOPs not containing key references
- Pregnancy assessment not documented during donor evaluation

More significant failures of compliance were less common. Examples include:
- Program not functioning as a single program (e.g. SOPs not uniform across different clinical sites, for example where allogeneic and autologous patients, or adult and paediatric patients were treated on different sites)
- Outpatient facilities inadequate (e.g. no provision for isolation of infectious patients)
- Inadequate quality management program
- No continuous temperature monitoring of freezers in processing facility
- Temperature not monitored during transport of HSC from processing facility to clinical unit
- Engraftment data not monitored by processing facility

Some of these significant deficiencies arose from lack of resources, e.g. size of laboratory inadequate for workload or lack of an experienced quality manager.

Analysis of Deficiencies
Deficiencies in the quality management program were by far the most common cause of failure of compliance with the standards and, including problems with policies and procedures (SOPs), accounted for 37% (636 of 1732) of total cited deficiencies (Figure 6). Deficiencies included:
- Problems with the formatting and content of SOPs, example:
  - missing examples of worksheets/forms/labels
  - missing references (where relevant)
  - failure to include range of expected results (where relevant)
- Lack of procedure for documenting deviations from SOPs
- Lack of regular review process for SOPs
- No SOPs for critical procedures e.g. bone marrow collection
- Inadequate document control procedure
- Lack of validation of equipment/procedures in collection and processing facilities
- Inadequate audit activity e.g.
  - no SOP for audit,
  - no written program for planned audits
  - no documentation of results of audits
  - no formal process for disseminating results
- Inadequate adverse event (AE) reporting/reviewing.

Centers often used a hospital-based incident reporting system, but in many cases it appeared from the number of reported AEs that this was not adequate to meet the needs of the HSCT program. Often it was not clear that all AEs were reviewed by the program director and/or that a report was issued to the patient’s physician.

Other significant problems included those related to donor selection and testing, labelling and process control. The commonest problems as documented for the clinical program, collection and processing facilities are summarised in Table 1. A list of common deficiencies is available on the JACIE website.

Centers often have problems in designing JACIE-compliant labels. This has been addressed by the International Cellular Therapy Coding and Labeling Advisory Group which has published terminology
special communication

and designed suitable labels for CTPs which are based on the ISBT 128 standard for stem cell component identification. An implementation plan has also been published by this group. Further information is available at http://iscbha.org/cellulartherapy_home.html. A Workshop of the EU Normalisation Committee (CEN) has agreed to recommend a modified version of the ISBT 128 system to the EU Directorate General for Public and Consumer Health (DG SANCO).

Current status of centers including time taken for correction /accreditation

Eighty-two centers have been awarded accreditation at least once. Of these, 58 are currently accredited including 4 reaccreditations and 7 of the remaining 10 centers whose accreditation has expired have presented themselves for reaccreditation (of the remaining 3 centers, one center has ceased transplantation and the other two centers have not yet requested reaccreditation). These 7 centers are among the 48 programs that are either awaiting reports, correcting deficiencies or who have presented evidence of corrections for assessment. One center abandoned the accreditation process but subsequently reapplied alongside a clinical unit.

The time taken by centers to present documentary evidence of corrections of deficiencies in 2007 varied from 18 to 297 days with an average of 130 days. In general the maximum amount of time allowed to correct deficiencies is 9 months (from the time the center receives the Accreditation Committee’s report.)

Experience of centers implementing JACIE

It was anticipated that implementation of the JACIE standards would pose some difficulties for applicant centers, particularly in relation to establishing a QM system and accompanying documentation. While QM is well established in laboratory practice, and most processing facilities will already have an established QM program, QM programs were rarely in place in clinical units. It was also anticipated that there would be resource implications in terms of staff time because of the amount of detailed documentation that is required to demonstrate compliance with the standards.

We undertook two surveys designed to assess the difficulties experienced by centers in preparing for accreditation. The results of these surveys have been published in detail before. Briefly, in the first survey we found that, in most centers, at least 18 months was needed for preparation. Twenty-two centers had additional staff other than the Program Director to manage project implementation, but these staff were only part-time in 13 centers and only 11 had any experience in quality management. The area of greatest difficulty for most centers was in the clinical program. Most difficulty was found in implementing the QM system, adverse event reporting system and other documentation. Some centers already had written policies and SOPs, an audit system and an adverse event reporting system, but in all cases further development was needed to bring these aspects of QM up to the required standard.

The results of the survey were consistent with the findings of the inspectors that the most common deficiencies were inadequacies in the QM system. Our findings also indicated that these arise from lack of trained staff and absence of QM culture, particularly in the clinical setting. There is clearly an important need for training of clinical staff (doctors and nurses) in quality management. It is also important for centers to have a designated quality manager who has appropriate experience in quality management systems.

In a second survey all responding centers indicated that they had benefited from implementing the JACIE standards. The areas of greatest perceived benefit were in procedure and practices, staff motivation, control of adverse events, and co-ordination between different areas of the program. Significant benefits were also perceived in patient satisfaction, facilities, patient care and safety and training of new and existing staff. The areas where little or no benefit was noted were in costs, compliance with requirements of health insurers/social security and in government recognition. It was evident that implementation and maintenance of a quality system increases the running costs of a program. Eighty-one percent of the centers reported that implementation of the QM system had highlighted a need for changes in the implementation of the transplant program and all felt that accreditation was worth the effort invested.


The requirements of Directive 2004/23/EC became law in EU member States on April 7th, 2006. The implementation of the parent Directive is supported by two Commission Directives (2006/17/EC and 2006/86/EC) which set out the detailed technical requirements. They cover (i) donation, procurement and testing, and (ii) coding, processing, preservation, storage and distribution and were published on February 8th 2006 and 24th October 2006, respectively. The current JACIE standards conform to the requirements of the Directive as regards donation, procurement and processing of stem cells, although JACIE is more detailed in many areas and JACIE standards also cover clinical transplant programs. However in some areas more ex-
The precise wording of the JACIE standards is required to fulfill the requirements of the Directive and appropriate changes were incorporated into the 3rd and 4th editions of the FACT-JACIE standards.

While support for accreditation among the professional transplant community is high, there are varying levels of engagement with JACIE by the regulatory authorities in different countries. It has proven very difficult to build up a standard picture of official support across the EU due to significant differences in regulatory structures, varying readiness to implement the Directive and political issues. However, it can be said that in a number of countries there has been support from the regulatory authorities, both direct and indirect, for the JACIE accreditation system. This is the case in Austria, Belgium, France, Italy, The Netherlands, and the United Kingdom.

In Spain, the National Transplant Organization (ONT) has signed a formal agreement with JACIE and national scientific societies which gives official support to voluntary accreditation by transplant units and will recognize accredited programs as meeting quality and safety requirements. (http://www.ont.es/contenido.jsp?id_nodo=306&keyword=&auditoria=F)

Outside the EU, a Swiss law on regulating transplants enacted in July 2007 directly cites JACIE in relation to HSC transplants and JACIE is cited as part of the law on compulsory health insurance requiring HSC centers to be certified by the Swiss Transplant Workgroup on Blood And Marrow Transplantation (STABMT) in accordance with JACIE Standards. All 10 HSC centers in the country have undergone inspection and the majority are now accredited.

Global harmonization of standards

JACIE, FACT, Netcord, AABB (formerly the American Association of Blood Banks) and the World Marrow Donor Association (WMDA) are working together to promote consistent interpretation of the requirements of the standards and guidance produced by each organization. A detailed comparison of the requirements of the EU Directives with the FACT-JACIE, Netcord-FACT, AABB and WMDA standards is being undertaken. It is a fundamental aim of JACIE to ensure that the FACT-JACIE standards as far as possible are harmonized with other applicable national and international requirements, including those of the EU. This is particularly important to prevent difficulties in importing and exporting tissues across international boundaries, which could occur if there were to be differences in the standards adopted in different countries.

One of the results of this collaborative approach has been the establishment of the Alliance for the Harmonisation of Cellular Therapy Accreditation (AHCTA) whose members include AABB, American Society for Blood & Marrow Transplantation (ASBMT); European Group for Blood & Marrow Transplantation (EBMT); Foundation for the Accreditation of Cellular Therapy (FACT); International Netcord Foundation; International Society for Cellular Therapy (Europe) (ISCT); Joint Accreditation Committee ISCT-EBMT (JACIE); and WMDA. AHCTA has developed a position paper on issues arising out of the import and export of HSC, together with a brief definition of the minimal standards that might be used to assess the provenance of imported HSC. The documents are accessible at www.ahcta.org. Within the EU discussions are now beginning on the establishment of a register of HSC collection centers and how this might be supported and hosted.

Conclusions

The FACT-JACIE accreditation system is now firmly established in Europe and the experience of centers that have been inspected is that implementation of the JACIE standards has led to significant improvements in different aspects of their transplant programs. JACIE has further assisted with a number of training courses for preparing centers for accreditation and has recently published a practical guide to quality management in transplant units. JACIE has also developed a close working relationship with other organizations involved in cellular therapy, which will form the basis for a new global approach to harmonization of standards and accreditation systems worldwide. This collaboration represents an innovative and proactive approach to solving the problems of international exchange of tissues and cells as these relate to the stem cell transplant community.

Acknowledgments

The authors would like to acknowledge the assistance of Prof Alois Gratwohl and Helen Baldomero for the data presented in Figure 5.
REFERENCES

1. Joint Accreditation Committee of ISCT (Europe) and the EBMT (JACIE). Available from: http://www.jacie.org
SPECIAL REPORT

Current status of JACIE accreditation in Europe: a special report from the Joint Accreditation Committee of the ISCT and the EBMT (JACIE)

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JACIE (Joint Accreditation Committee of the ISCT and the EBMT) launched its first official inspection programme in January 2004. Since then, 35 centres in Europe have been inspected. Almost all were found to be functioning at a high level of excellence, with the majority having only minor deficiencies in compliance with the standards. In one-third of centres there were more significant deficiencies. The most common deficiencies were in quality management, and a survey of the applicant centres confirmed this was the area where centres experienced most difficulty in preparation for accreditation. Following correction of deficiencies, 28 centres have at the time of writing achieved full accreditation. Implementation of JACIE required a significant investment of time and resources by applicant centres. The majority required at least 18 months to prepare for accreditation and 85% needed to employ a quality manager and/or data manager on an ongoing basis. However, all centres felt their programme had benefited from the implementation of JACIE. In addition to the inspection and accreditation of individual centres, JACIE maintains an educational programme including training courses for inspectors and for centre preparation. JACIE is also working closely with other international organisations working in cellular therapy to develop international standards for all aspects of stem cell transplant. The recent implementation of Directive 2004/23/EC has provided an impetus for the implementation of JACIE in EU member states and in particular the requirements for safety of imported tissues and cells have emphasised the need for global harmonisation.

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Keywords: transplant; accreditation; standards; inspection; regulation

Introduction

JACIE is a non-profit body established for the purposes of assessment and accreditation in the field of haematopoietic stem cell transplantation (HSCT). The committee was founded in 1998 by the European Group for Blood and Marrow Transplantation (EBMT) and the International Society for Cellular Therapy (ISCT), the two leading scientific organisations involved with HSCT in Europe. JACIE modelled itself on the US-based Foundation for the Accreditation of Cellular Therapy (FACT), established in 1996 by the ISCT and the American society for Blood and Marrow Transplantation (ASBMT). JACIE actively collaborates with FACT in establishing standards for the provision of quality medical and laboratory practice in HSCT. JACIE conducts inspections, accredits programmes and encourages health institutions and facilities performing HSCT to meet these standards voluntarily in order to demonstrate their high levels of quality of care. The current organisation of JACIE is shown in Figure 1. The structure ensures wide consultation, with 20 European countries now represented on the Board in addition to nursing, paediatrics and cord-blood representatives.

The primary aim of JACIE is to improve the quality of HSCT in Europe by providing a means whereby transplant centres, cell collection facilities and processing facilities can demonstrate high-quality practice. This is supported by coordinating training courses in quality management for applicant centres and courses for inspectors. An additional and wider aim is to ensure harmonisation between JACIE standards and other national/international standards, including the EU Tissues & Cells Directive (Directive 2004/23/EC) and the related implementing Directives. The increasing use of unrelated donor cells for transplants highlights the need for further work in this area.

Accreditation of HSCT facilities is through online submission of documentation and an on-site visit by a team of trained inspectors. Centres may apply for accreditation as complete programmes comprising a clinical programme, a collection facility and a processing laboratory or, for example, as a single collection or processing facility serving a number of clinical programmes. JACIE accreditation is voluntary, but provides a means whereby transplant facilities can demonstrate that they are working within a quality system covering all aspects of the
transplantation process and thus show compliance with the requirements of insurance companies or national and/or international regulatory authorities.

Following a programme of four pilot inspections in Spain between 2001 and 2003, where FACT inspectors performed the first on-site visit, the JACIE programme was fully implemented in January 2004 with support from the European Union under the Public Health Programme (2003-2008). Between January 2004 and August 2006, 35 centres were inspected. These included two in Austria, two in Finland, six in France, one in Italy, five in The Netherlands (an additional Dutch centre was inspected in 2004 as part of national pilot project but this centre was not the subject of a formal accreditation review), 10 in Switzerland and nine in the UK. This experience has enabled JACIE to identify areas of common difficulty for applicant centres, to assess what assistance centres need in order to achieve accreditation, and has also raised some general issues relating to national and international regulation.

The JACIE standards

The JACIE standards cover all aspects of clinical transplant programmes, collection facilities (BM collection and peripheral blood progenitor cell collection) and processing, as shown in Table 1. The standards also apply to the use of therapeutic cells derived from blood or marrow, including donor lymphocytes.

Within each subsection are detailed lists of specific standards; for example the standard on donor evaluation and selection contains 33 specific items relating to clinical evaluation, laboratory testing, informed consent, etc. The complete standards and the accompanying guidance manual are available on the JACIE website. Work on the third edition of the standards is now complete and they will be released shortly.

JACIE standards conform to the Tissues and Cells Directive as regards donation, procurement and processing of stem cells, but also cover clinical transplant programmes. Accreditation of clinical programmes includes the clinical use of cord blood (CB) stem cells but the JACIE programme does not accredit CB collection and banking facilities as this process is currently carried out by Netcord-Fact.

The JACIE manual and inspection checklist

The manual contains the standards together with detailed guidance on the interpretation and measures required to demonstrate compliance. Each standard is followed by specific questions relating to that standard and these questions form the basis of the inspection checklist, which must be completed before inspection by the applicant centre and verified by the inspector during the inspection (Figure 2).

The accreditation process

Preparation by centre

The centre implements measures as described in the JACIE accreditation manual, and then applies for inspection by submitting basic information about the programme/facility and a number of supporting documents including a

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**Table 1** Contents of the JACIE Standards (2nd ed. June 2003)

<table>
<thead>
<tr>
<th>Clinical programme</th>
<th>Collection</th>
<th>Processing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size and organisation</td>
<td>Facilities</td>
<td>Facilities</td>
</tr>
<tr>
<td>Staffing</td>
<td>Staffing</td>
<td>Staffing</td>
</tr>
<tr>
<td>Quality Management Programme</td>
<td>Quality Management Programme</td>
<td>Quality Management Programme</td>
</tr>
<tr>
<td>Donor evaluation and selection</td>
<td>Donor evaluation/care at time of collection</td>
<td>Donor evaluation/care at time of collection</td>
</tr>
<tr>
<td>Administration of high dose therapy</td>
<td>Collection procedure (BM or PBPC)</td>
<td>Collection procedure (BM or PBPC)</td>
</tr>
<tr>
<td>Clinical research</td>
<td>Labels</td>
<td>Labels</td>
</tr>
<tr>
<td>Data management</td>
<td>Records</td>
<td>Records</td>
</tr>
</tbody>
</table>

Abbreviations: BM, bone marrow; PBPC, peripheral blood progenitor cells.

---

Bone Marrow Transplantation
### Table: Inspection Checklist

<table>
<thead>
<tr>
<th>B6.000 DONOR EVALUATION, SELECTION AND MANAGEMENT</th>
<th>APPLICANT</th>
<th>INSPECTOR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B6.100 Are there donor evaluation procedures in place to protect the safety of the haematopoietic progenitor cell donor and recipient?</strong></td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td><strong>Do these evaluation procedures assess the potential for disease transmission from the donor to the recipient?</strong></td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td><strong>Do these evaluation procedures assess the risks to the donor from the collection procedure?</strong></td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td><strong>Are donor evaluation and selection test results documented?</strong></td>
<td>D</td>
<td>Y</td>
</tr>
<tr>
<td><strong>B6.110 Are there written criteria for donor evaluation and selection?</strong></td>
<td>D</td>
<td>Y</td>
</tr>
</tbody>
</table>

Figure 2  Example from checklist. D = deficiency if answered 'No' by applicant.

---

self-assessment checklist. The application information and checklist must be submitted in English, but all other documentation, including Standard Operating Procedures (SOPs), is accepted in the language of the centre.

**Inspection**

An on-site visit is carried out by a team of trained inspectors, usually one per facility (clinical/collection/processing). Inspectors are medical, scientific or other professional persons working in HSCT, with specific qualifications and experience for inspecting clinical, collection and/or processing facilities. Inspectors must attend a JACIE-sponsored training course and pass an examination. Where a clinical programme performs adult and paediatric transplants, an adult and a paediatric inspector will be assigned. Inspectors may also be from another country, but should be either native or fluent speakers of the relevant language. In countries where it is not possible to assign an inspector who speaks the language, a local expert is requested to assist with translation of interviews and documents as necessary.

An inspection visit lasts 1–1.5 days and involves discussion with staff during their work, review of documents/records and completion of a detailed checklist relating to the standards. The inspectors write a report in English, noting any areas of non-compliance with the standards, which is reviewed by the JACIE Office. Based on the inspectors’ findings, a supplementary report from the JACIE Medical Director (MD) is prepared indicating the current level of compliance (Table 2) and making specific recommendations for corrections and improvements. The distinction between minor deficiencies and more significant deficiencies is not strictly defined, but in general terms, specific deficiencies in documentation are considered minor whereas more general problems with documentation or problems with processes or facilities are considered significant. Between 3 and 12 months is allowed for the centre to correct deficiencies, depending on the amount of work required. The Inspection report and Medical Director’s (MD) report are approved by the JACIE Executive Committee and then issued to the applicant centre. Recently, the JACIE Accreditation Committee was established, which will take over this role.

**Centre response**

The centre must indicate acceptance of the findings and then in due course submit documentary evidence to confirm corrections or amendments. The original inspectors review the documentation. Review by the inspectors rather than by the Accreditation Committee, MD or JACIE office alone is required because of language issues. In some cases, a limited revisit may be the best way to show that deficiencies have been remedied. The inspectors confirm to JACIE that all necessary corrections have been made or indicate that there are still outstanding areas for completion.

**Accreditation**

The Medical Director reviews all the reports and relevant documentation and if satisfied that all previous deficiencies have been corrected, the JACIE Executive Committee (in future the JACIE Accreditation Committee) makes a recommendation to the JACIE Board that the centre be awarded accreditation. If approved, accreditation is awarded, valid for 3 years, subject to an annual report from the centre noting any significant changes in personnel or procedures and including annual activity figures.
Table 2  Levels of compliance

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No deficiencies or variances from recommendation observed at the on-site inspection or documented on submitted materials. Full accreditation for 3 years awarded effective from the date of the Board decision.</td>
</tr>
<tr>
<td>2</td>
<td>Few minor deficiencies noted at the on-site inspection and/or documented in submitted materials. Full accreditation requires Programme Director’s documentation of correction of all deficiencies and satisfactory response to recommendations.</td>
</tr>
<tr>
<td>3</td>
<td>Significant defect or deficiencies documented at the site inspection. Full accreditation requires Programme Director’s documentation of correction of all deficiencies and satisfactory response to recommendations.</td>
</tr>
<tr>
<td>4</td>
<td>Significant defect or deficiencies observed at the site inspection. Full accreditation requires Programme Director’s documentation of correction of all deficiencies and satisfactory response to recommendations. Documentation of correction of deficiencies also requires that a focused re-inspection of one or more areas of the facility operation be conducted.</td>
</tr>
<tr>
<td>5</td>
<td>Significant deficiencies observed during the site inspection requiring a full re-inspection of the applicant facility to document correction of all deficiencies.</td>
</tr>
<tr>
<td>6</td>
<td>Nonaccreditation. Reapplication and submission of documents required.</td>
</tr>
</tbody>
</table>

Since 2006 the numerical gradings are used only by JACIE for internal purposes and do not form part of reports to centres.

Outcome of inspections

To date, over 80 facilities have formally applied for accreditation. Between January 2004 and August 2006 35 centres were inspected. Twenty-nine centres applied for accreditation for a combination of clinical, collection and processing facilities. One centre applied for clinical and collection only, three centres applied for collection and processing, one for apheresis collection only and one for processing only.

Almost all were found to be functioning at a high level of excellence. Fifty-seven per cent were assessed as initially compliant at level 2, 40% at level 3, and 3% at level 4. In four centres assessed at level 3 a limited re-inspection was recommended as the simplest means of demonstrating compliance because of general problems in the quality management system (QMS) of the clinical programme.

The most common deficiencies were in documentation, labelling and in the quality management programme. This is consistent with the initial experience of the FACT accreditation programme in the United States.

Common deficiencies

Minor failures of compliance were frequent, usually involving problems with documentation. Examples include:

- SOPs missing references.
- Pregnancy assessment not documented during donor evaluation.

More significant failures of compliance were less common. Examples include:

- Programme not functioning as a single programme (e.g. SOPs not uniform across different clinical sites, for example, where allogeneic and autologous patients, or adult and paediatric patients were treated on different sites).
- Outpatient facilities inadequate (e.g. no provision for isolation of infectious patients).
- Inadequate QMS.
- No continuous temperature monitoring of freezers in processing facility.
- Temperature not monitored during transport of cells from processing facility to clinical unit.
- Engraftment data not monitored by processing facility.

Some of these significant deficiencies arose from lack of resources, for example, size of laboratory inadequate for workload or lack of an experienced quality manager.

Analysis of deficiencies

QMS. Deficiencies in the QMS were by far the most common cause of failure of compliance with the standards and, including problems with policies and procedures (SOPs), accounted for 35% (201 of 570) of total cited deficiencies (Figure 3).

Deficiencies in QMS included:

- Problems with the formatting and content of SOPs, for example,
  - missing examples of worksheets/forms/labels,
  - missing references (where relevant) and
  - failure to include range of expected results (where relevant).
- Lack of procedure for documenting deviations from SOPs.
- Lack of regular review process for SOPs.
- No SOPs for critical procedures, for example, bone marrow collection.
- Inadequate document control procedure.
- Lack of validation of equipment/procedures in collection and processing facilities.
- Inadequate audit activity, for example,
  - no SOP for audit,
  - no written programme for planned audits,
  - no documentation of results of audits and
  - no formal process for disseminating results.
- Inadequate adverse event (AE) reporting/reviewing. Centres often used a hospital-based incident reporting system, but in many cases it appeared from the number of reported AEs that this was not adequate to meet the needs of the HSCT programme. Often it was not clear that all AEs were reviewed by the programme director and/or that a report was issued to the patient's physician.
**Patient/donor issues**

Deficiencies in documentation were frequent.

- No verification of patient's initial diagnosis from primary records.
- No formally documented criteria for defining suitable donor.
- Failure to document vaccination history, transfusion history or travel history.
- Pregnancy not always assessed in female donors of childbearing age.

**Labelling of components**

JACIE standards for labelling of components require detailed information to be shown on the label. In the majority of centres, it was found that labelling of products during collection and processing was not compliant with the standards in a variety of ways, accounting for 15% of all cited deficiencies (83 of 570), for example,

- time of end of collection/processing missing,
- volume of anticoagulant missing, and
- unique identifier (alphanumeric number) not used at collection.

It appears that centres have a common problem in designing JACIE-compliant labels. This is being addressed by the International Cellular Therapy Coding and Labeling Advisory Group, which is aiming to design suitable labels for general use and incorporating the requirements of the ISBT 128 standard for stem cell component identification and has provisionally set May 2007 as the date by which to complete its final documents and recommendations. Further information on the work of this group is available at http://iccbba.org/cellulartherapy_home.html.

The analysis of common deficiencies in Figure 3 shows that quality management and component labelling are the most frequently observed sources of deficiencies. Incomplete donor evaluation was also frequent. The frequency of deficiencies involving quality management and AE reporting are consistent with the reported difficulties experienced by centres (see below). A list of common deficiencies is available on the JACIE website.

**Current status of centres including time taken for correction/accreditation**

At the time of reporting 28 of the 35 inspected centres have completed correction of all deficiencies and achieved accreditation with a further four recommended to the Board for accreditation, as shown in Table 3. One additional clinical programme is awaiting accreditation of...
Table 3  JACIE Accredited Centres – August 2006

<table>
<thead>
<tr>
<th>Country</th>
<th>Institution (Address)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>St Anna Kinderklinik, Vienna</td>
</tr>
<tr>
<td>Austria</td>
<td>First Medical Department, Medical University of Vienna</td>
</tr>
<tr>
<td>Finland</td>
<td>Helsinki University Central Hospital</td>
</tr>
<tr>
<td>France</td>
<td>Service des Maladies du Sang, Hôpital Haut-Lévêque, Bordeaux</td>
</tr>
<tr>
<td>France</td>
<td>Service d’Hématologie, Hôpital E Herriot, Lyon</td>
</tr>
<tr>
<td>France</td>
<td>Fédération de Greffe de Moelle et de Thérapie Cellulaire d’Auvergne, Clermont Ferrand</td>
</tr>
<tr>
<td>France</td>
<td>Service d’Hématologie Clinique du CHU Henri Mondor et Laboratoire</td>
</tr>
<tr>
<td>France</td>
<td>Prélèvements thérapeutiques de l’EFS (Site Henri Mondor), Creteil</td>
</tr>
<tr>
<td>France</td>
<td>Institut Gustave Roussy, Département de Pédiatrie, Villejuif</td>
</tr>
<tr>
<td>France</td>
<td>Service d’Hématologie-Greffe de moelle, Hôpital St Louis, Paris</td>
</tr>
<tr>
<td>Italy</td>
<td>Department of Pediatrics, Ospedale Regina Margherita di Torino</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Leids Universitair Medisch Centrum, Leiden</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Department of Haematology, HaagZiekenhuis, The Hague</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Erasmus Medical Centre, Rotterdam</td>
</tr>
<tr>
<td>Netherlands</td>
<td>UMC Utrecht Stem Cell Laboratory</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Kantonsspital, Aarau</td>
</tr>
<tr>
<td>Switzerland</td>
<td>University hospital, Basel</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Istituto Oncologie della Svizzera Italiana, Bellinzona</td>
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<tr>
<td>Switzerland</td>
<td>SZT – Program, Universitätsklinik, Bern</td>
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<tr>
<td>Switzerland</td>
<td>Service d’Hématologie, Hôpital Cantonal Universitaire, Geneva</td>
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<tr>
<td>Switzerland</td>
<td>Programme lausannois de transplantation de cellules souches hématoïdipoides autologues, Lausanne</td>
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<tr>
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<td>Kantonsspital, St Gallen</td>
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</table>

Please refer to www.jacie.org for the most up-to-date list of accredited centres (EFS: Établissements Français du Sang; NBS: National Blood Service).

their human leukocyte antigen typing laboratory by the European Federation of Immunogenetics EFI (a requirement under standard B2.190) before full accreditation can be awarded but is otherwise fully compliant with the JACIE standards. Five programmes are still in the process of correcting deficiencies. The time taken to document correction of deficiencies has varied from 5.5 to 19 months, with a median of 11.5 months. In future, the maximum amount of time allowed to correct deficiencies will be 12 months (from the time the centre receives the initial report).

Experience of centres implementing JACIE.

It was anticipated that implementation of the JACIE standards would pose some difficulties for applicant centres, particularly in relation to establishing a QMS and accompanying documentation. Although QM is well established in laboratory practice, and most processing facilities will already have an established QM programme, a QM programme is rarely in place in clinical units. It was also anticipated that there would be resource implications in terms of staff time because of the amount of detailed documentation that is required to demonstrate compliance with the standards.

An initial survey was designed to assess the difficulties experienced by centres in preparing for accreditation. All centres were asked to complete the survey immediately following inspection and return to the JACIE Office. The survey was completed online and respondents were given the choice of including their centre name or submitting the survey anonymously.

The survey included questions addressing the following issues:

- Motivation for applying.
- Time period from decision to apply to date of inspection.
- Level and experience of staffing responsible for project management.
- Part of programme (clinical, collection, processing) where most difficulty was experienced.
- Level of difficulty experienced in different areas (e.g., implementing QMS, training).
- Extra resources specifically required to implement JACIE.
- Impact of implementation of JACIE in terms of the functioning of the programme.

A second survey was designed to assess the effect of implementation of JACIE. Centres were asked to complete this survey 6 months after inspection. This survey asked for an estimate of the number of additional staff hours per week required for maintaining the JACIE Standards and sought the centres’ views on the benefit or drawbacks of having implemented JACIE. This survey included questions addressing the following issues:

- Whether programme had benefited from implementation of JACIE and if so in what areas.
Did implementation of QMS bring to light any need for changes in the implementation of the transplant programme, and if so in what areas.

- Ongoing resource requirements to maintain standard.
- Overall opinion on value of JACIE implementation.

Survey results
Twenty-six centres have so far returned Survey 1 and 22 returned Survey 2.

Survey 1: common difficulties
The results of the survey were as follows:

- At least 18 months is needed for preparation in the majority of cases (6 months 4%; 1 year 19%; 18 months 42%; 2 years 31%).
- Twenty-two centres had additional staff other than the Programme Director to manage project implementation, but these staff were only part-time in 13 centres and only 11 had any experience in quality management.
- The area of greatest difficulty for most centres (Table 4) was in the clinical programme (clinical programme 62%, bone marrow collection facility 42%, peripheral blood stem cells collection facility 12%, processing facility 15% (some centres ticked more than one choice).
- Most difficulty was found in implementing the QMS, AE reporting system and other documentation. Twenty-four centres already had written policies and 88% had SOPs, an audit system and an AE reporting system, but in all cases further development was needed to bring these aspects of QMS up to the required standard. Lack of a culture of QM was cited as an important problem.
- The extra resources most frequently required were a quality manager (62% centres) and a data manager (35%). Only 19% needed to improve their facilities.
- Some financial support was provided in addition to departmental budget in 69% cases, either from hospital resources (35%), external grant (27%) or, in one case, from pharmaceutical companies.

The results of the survey are consistent with the findings of the inspectors that the most common deficiencies are inadequacies in the QMS. The survey also indicates that these arise from lack of trained staff and absence of QM culture, particularly in the clinical setting. There is clearly an important need for training of clinical staff (doctors and nurses) in quality management. It is also important for centres to have a designated quality manager who has appropriate experience in QMS. One of the major aims of JACIE over the next 1-2 years is provision of more educational material such as model documents and a guide to implementing QMS in a transplant centre.

Survey 2: Impact of implementing JACIE
All responding centres indicated that they had benefited from implementing the JACIE standards. As shown in Table 5, the areas of greatest perceived benefit were in procedure and practices, staff motivation, control of AE and coordination between different areas of the programme. Significant benefits were also perceived in patient satisfaction, facilities, patient care and safety and training of new and existing staff.

Improvements clearly depend on the level of existing services, so that failure to demonstrate improvement in, for example, facilities or data management may reflect good pre-existing resources. In other areas, for example, AE reporting, the systems for monitoring performance were only set up as part of implementing JACIE, so that it is difficult to monitor improvements without an established baseline for comparison. Indeed implementation of JACIE may have the paradoxical effect of seeming to increase AEs because these were not previously adequately reported.

Nevertheless it seems clear that there are definite benefits in many areas of the programme, with a high degree of consistency in replies. The areas where little or no benefit was noted were in costs, compliance with requirements of health insurers/social security and in government recognition. It is evident, as discussed below that implementation and maintenance of a quality system increases the running costs of a programme. Regarding compliance with requirements of health insurers/social security and government

| Table 5: Effect of implementation of JACIE |

<table>
<thead>
<tr>
<th>Level of improvement</th>
<th>High (%)</th>
<th>Medium (%)</th>
<th>Low (%)</th>
<th>None (%)</th>
<th>NA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control of incidents, events and adverse reactions</td>
<td>41</td>
<td>59</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Data management</td>
<td>23</td>
<td>64</td>
<td>9</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Internal coordination</td>
<td>50</td>
<td>32</td>
<td>9</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Patient satisfaction</td>
<td>23</td>
<td>36</td>
<td>18</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Staff motivation</td>
<td>55</td>
<td>45</td>
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<td></td>
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</tr>
<tr>
<td>Cost</td>
<td>9</td>
<td>18</td>
<td>23</td>
<td>45</td>
<td>5</td>
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<tr>
<td>Facilities</td>
<td>50</td>
<td>18</td>
<td>32</td>
<td></td>
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<tr>
<td>Patient safety and care</td>
<td>27</td>
<td>55</td>
<td>9</td>
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<tr>
<td>Procedures and practices</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Training of new/existing staff</td>
<td>41</td>
<td>45</td>
<td>9</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Compliance with health insurers/social security demands</td>
<td>14</td>
<td>9</td>
<td>23</td>
<td>45</td>
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<tr>
<td>Government recognition</td>
<td>18</td>
<td>27</td>
<td>27</td>
<td>23</td>
<td>5</td>
</tr>
</tbody>
</table>

Level of improvements in programme functioning experienced by centres, as reported in Survey 2.
Results are expressed as percentage of responding centres. NA: this question not answered.

Abbreviations: JACIE, Joint accreditation committee ISCT-EBMT; QMP, quality management plan.
Results are expressed as percentage of responding centres. NA: this question not answered.
recognition, this is likely to change in the near future with the impact of the tissues and cells Directive on the requirement to implement inspection and accreditation programmes in member states.

Eighty-one per cent of the centres reported that implementation of the QM system had highlighted a need for changes in the implementation of the transplant programme. The most common areas cited were a need for improved coordination between the different facilities, that is, clinical, collection and processing, and a need for more systematic audit and AE reporting. Other items were a need to improve patient medical records and to improve donor evaluation procedures.

All centres felt that accreditation was worth the effort invested. The majority (59%) replied “The effort is a huge strain on resources but on balance it is worth it” whereas eight said “There is no doubt that accreditation is worth the effort invested” (one failed to comment). With the implementation of the EU Directive on safety of tissues and cells (Directive 2004/23/EC) it is likely that collection and processing facilities will increasingly view compliance with JACIE standards as important in providing evidence that they are complying with the requirements of the Directive.

Ongoing resource requirements

All centres reported a requirement for additional resources on an ongoing basis. Eighty-two per cent (18/22) of the centres needed to employ additional staff on a permanent basis. Sixteen centres employed a new quality manager, full time in two cases and part-time in 14. One centre employed an additional medical staff and one employed a full-time transplant coordinator. Eighteen centres cited a need for ongoing training and extra effort by staff and seven centres reported using external consultation and independent audit as a means of improving their programme. Ongoing funding was obtained from departmental budgets and in many cases also hospital resources.

Comment on survey results

Despite the difficulties experienced by centres in implementing JACIE, there is no doubt that they felt the effort was worth it and that their programme had benefited from the process. There is also no doubt that additional resources are needed on an ongoing basis once the standards have been implemented. In the future there is a need to assess the effect of implementation on a more formal basis, using for example analyses of AEs and patient satisfaction questionnaires in order to allow a cost–benefit analysis of JACIE implementation. The resource implications experienced at one centre were reported in detail by Zahnd et al.9

Implications of EU Directive 2004/23/EC

The requirements of the Directive became law in EU member States on 7 April 2006. In order to support implementation of the Directive, two technical annexes have been drawn up by the EU, which set out the detailed technical requirements of the Directive. The first covers donation, procurement and testing, and the second coding, processing, preservation, storage and distribution. Technical Annex 1 of the Directive was published as Commission Directive 2006/17/EC on 8 February 2006 and Technical Annex 2 was published as Commission Directive 2006/86/EC on 24 October 2006. Updated information will be provided by the European Legal and Regulatory Affairs Committee on the ISCT website (http://www.celltherapy-society.org).

The current JACIE standards conform to the requirements of the Directive as regards donation, procurement and processing of stem cells, although JACIE is more detailed in many areas and JACIE standards also cover clinical transplant programmes. However, in some areas more explicit wording of the JACIE standards is required to fulfil the requirements of the Directive and appropriate changes have been incorporated into the third edition of the FACT–JACIE standards.

JACIE, FACT and the World Marrow Donor Association (WMDA) are working together to promote consistent interpretation of the requirements of the Directive by the regulatory authorities in the different member states. A detailed crosswalk comparing the requirements of the EU Directive and technical annexes with the FACT–JACIE standards and WMDA guidelines is in preparation (details will be available on the JACIE website).

Although support for accreditation among the professional transplant community is high, there are varying levels of engagement with JACIE by the regulatory authorities in different countries. It has proven very difficult to build up a standard picture of official support across the European Union owing to significant differences in regulatory structures, varying readiness to implement the Directive and political issues. However, it can be said that in a number of countries there has been support from the regulatory authorities, both direct and indirect, for the JACIE accreditation system. This is the case in Austria, Belgium, France, Italy, The Netherlands and the United Kingdom. In Spain, the National Transplant Organisation (ONT) has signed a formal agreement with JACIE and national scientific societies, which gives official support to voluntary accreditation by transplant units and will recognise accredited programmes as meeting quality and safety requirements. 31-10-2006 http://actualidad.terra.es/sociedad/articulo/ont_impulsa_acreditacion_centros_transfusion_1178645.htm).

Outside the EU, Swiss national law on regulating transplants already directly cites JACIE in relation to HSC transplants. A new law is currently being drafted that will require all HSC transplant centres to be JACIE accredited with effect from 1 January 2007. JACIE is currently cited as part of the law on compulsory health insurance requiring H SCT centres to be certified by the Swiss Transplant Group with Blood And Marrow Transplantation in accordance with JACIE Standards. All 10 HSC centres in the country have undergone inspection and the majority are now accredited.
Global harmonisation of standards

It is a fundamental aim of JACIE to ensure that the FACT-JACIE standards as far as possible are identical to other applicable national and international requirements, including those of the EU. This is particularly important to prevent difficulties in importing and exporting tissues across international boundaries, which could occur if there were to be differences in the standards adopted in different countries.

JACIE has worked together with FACT on the 3rd edition of the standards and guidance, a draft of which was made available for public consultation in February 2006 and which is expected to be published in late 2006. The new version will be issued as a joint FACT-JACIE document. JACIE and FACT are also working with national and international donor registries and the WMDA to promote the use of international standards for collection facilities for unrelated donor transplants wherever possible, without jeopardising the availability of HSC from unrelated donors.

One of the results of this collaborative approach has been the establishment of an Alliance for the Harmonisation of Cellular Therapy Accreditation, whose members include American Association of Blood Banks, ASBMT, EBMT, FACT, International NETCORD Foundation, ISCT (Europe), JACIE and WMDA.

Conclusion

The JACIE accreditation system is now firmly established in Europe and the experience of centres that have been inspected is that implementation of the JACIE standards has led to significant improvements in different aspects of their transplant programmes. JACIE has further assisted with a number of training courses for preparing centres for accreditation and is currently working on a practical guide to quality management in HPC units. JACIE has also developed a close working relationship with other organisations involved in cellular therapy, which will form the basis for a new global approach to harmonisation of standards and accreditation systems worldwide. This collaboration represents an innovative and proactive approach in solving the problems of international exchange of tissues and cells as these relate to the stem cell transplant community.

Acknowledgements

In 2004, the European Commission part-funded the JACIE Project under the Programme of Community action in the field of public health (2003-2008). The total project budget of €351,695 was also supported by the EBMT and ISCT, and a number of national haematology and HSCT societies. We would like to acknowledge the assistance of Sarah Craig, JACIE administrative officer, and Sara Notley, EBMT administrative officer in the work of JACIE and the preparation of this paper.

References

bone marrow and peripheral blood stem cell donors who were recruited, selected, harvested, and followed at our institution and the associated donor registry. 40% were females and 60% males, with a median age of 37 years (range: 17-71 years). 318 were family donors and 278 unrelated volunteer donors. 110 donated bone marrow, 472 peripheral blood stem cells after mobilisation with G-CSF and 14 both. 39 donors donated for two harvests, four donors even three. Our preparative procedure consisted of a medical work-up including history, examination, blood and clinical chemistry tests, infectious disease markers, electrocardiogram, chest X-ray, lung function test, and abdominal ultrasound. Follow-up was scheduled at 1 and 6 months, as well as 1, 2, 5, and 10 years after donation of allogeneic stem cells. Results. Within the twelve year period of evaluation, no donor death was recorded, no single case of leukemia has been reported, and no splenic rupture occurred in any of our donors. Five donors experienced severe adverse events: one female bone marrow donor, post-harvesting pain persisted for more than twelve months. A 42 year old female donor developed breast cancer 3 years after G-CSF mobilised stem cell apheresis; however, this was not considered to be of causation relationship. In two donors of peripheral blood stem cells, vascular complications occurred: one donor had to be admitted to hospital due to a perforation of the iliac vein with subsequent ileus which, however, could be ruled out, and subsequently was able to donate bone marrow. Another donor had occurrence of a deep venous thrombosis eleven days after stem cell apheresis, despite sufficient anticoagulation with low molecular weight heparin during G-CSF mobilisation. Finally, a donor with sporadic epilepsy who donated twice, had a generalised seizure few days after each G-CSF mobilised harvest. This is particularly interesting in view of recent in vitro and animal findings of G-CSF receptors on neural tissues and pleiotropic neuroregulatory functions of G-CSF. Conclusions. In our experience, bone marrow and peripheral blood stem cell donors are safe and generally well tolerated, with very few early and virtually no late complications.

0369 ALLOGENEIC STEM CELL TRANSPLANTATION FOR PATIENTS WITH WALDENSTRUM'S MACROGLOBULINAEMIA. ANALYSIS OF 106 CASES FROM THE EUROPEAN BONE MARROW REGISTRY (EBMT)

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1University College London, LONDON; 1Lymphoma WP of the EBMT, LONDON, United Kingdom

Background. Despite effectiveness of standard chemotheraphy regimens, complete response is infrequent in Waldenström's Macroglobulinaemia (WM) patients and there is no cure. The role of allogeneic stem cell transplantation (Allo-SCT) has not been explored extensively and the available data are limited. Aim. We retrospectively analyzed the results and long-term outcome of a group of 106 WM patients from 65 European centres who underwent an Allo-SCT between 1989 and 2005 and were reported to the database of the Lymphoma WP of the EBMT. Patients and Methods. There were 69 males and median age at transplantation was of 49 years (range: 21-66). Time interval between diagnosis and Allo-SCT was 34 months (0-310) and the median number of lines of therapy prior to Allo-SCT was 3. Nineteen patients (18%) had a failed prior autograft. At Allo-SCT, 10 patients (10%) were in CR1, 29 patients (34%) in PR1, 29 patients (34%) in PR2 and 18 patients (20%) in PR3. Forty-eight (45%) patients developed advanced B-cell chronic lymphocytic leukemia. Seventy-nine patients (74%) were allografted from an HLA-identical sibling donor, 18 (17%) from a matched unrelated donor and the remaining 9 patients from other donors. Conventional conditioning protocols (CT) were used in 44 (41%) patients and reduced intensity conditioning (RIC) regimens in 62 (59%) patients. Results. Forty-eight (45%) patients died, 5 (5%) from disease progression and 30 (28%) from non-relapse mortality (NRM), with an incidence of NRM of 27% and 31% at 1 and 3 years. The progression free survival at 3 years was 19%, 12% after CT and 25% after RIC. Thirty-five (35%) patients died, 5 (5%) from disease progression and 30 (28%) from non-relapse mortality (NRM), with an incidence of NRM of 27% and 31% at 1 and 3 years. The progression free survival at 3 years was 19%, 12% after CT and 25% after RIC. Thirty-five (35%) patients died, 5 (5%) from disease progression and 30 (28%) from non-relapse mortality (NRM), with an incidence of NRM of 27% and 31% at 1 and 3 years. The progression free survival at 3 years was 19%, 12% after CT and 25% after RIC.
The South African Bone Marrow Registry (SABMR): a 10-year review

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²Department of Haematology and Bone Marrow Transplant Unit incorporating the Searl Laboratory for Molecular and Cellular Biology, Constantiaberg Medi-Clinic, Cape Town

The initiative to establish the SABMR in 1991 came from the Laboratory for Tissue Immunology via Professor Ernette du Toit and the Department of Haematology at Groote Schuur Hospital under the headship of Professor Peter Jacobs in association with the Western Province Blood Transfusion service directed by Dr. Arthur R Bird. The SABMR was designated as the Hub centre for South Africa (SA) by the World Marrow Donor Association (WMDA) in October of the same year. Much research was being carried out by the LTI in the field of HLA typing with particular reference to population genetics. These results were the raison d'être for starting this facility that was rapidly expanded to accommodate the inclusion of unrelated donors.

The SABMR is responsible for co-ordinating the provision of HLA-compatible matched unrelated volunteers (MUD) in this country and is the point of contact beyond these borders for seeking such donors. The latter must be between the ages of 18–55 years of age as well as being healthy and committed. By the end of 2001, there were 20,000 HLA typed people on file with these having been recruited with the help of two sub-registries respectively designated the South African and Natal Blood Transfusion Service (SABTS and NBTS). This data is registered with the Bone Marrow Donor World-wide (BMDW) and thus the SABMR donors form part of the >7 million individuals in 49 adult and 25 cord blood listings. Preliminary identification via this mechanism for people in South Africa increased from ±100 in 1992 to ±300 in 2001 with the reverse pattern increasing from 3 in 1992 to 48 in 2001. A donor search has correspondingly risen from 7 in 1998 to a total of 58 by 2001.

WMDA protocols are meticulously observed, dealing, inter alia, with registration, confidentiality, counselling and the transport of grafts. Responsibilities of the Hub centre have also been documented with regard to the patient demographics, searches, review of results, as well as financial and legal liabilities. When considering unrelated individuals a prerequisite is that HLA matching be as close as possible and DNA class I and II is now standard methodology. The first local MUD transplant took place in 1997 and by 2001 a total of 25 patients had received similar grafts (see Fig. 1). In order to retain international status accreditation for the Laboratory is mandatory and was achieved in 1999.

The majority of the patients received bone marrow from international registries, see Fig. 2. The numbers denote the number of protocols received, which include bone marrow, peripheral blood stem cells (PBSC) and donor lymphocyte infusions (DLI). A further step in the growth of the SABMR, was the identification of local donors for SA patients. The first such transplant took place in Cape Town in 1999, and since then a further four local donors have been used.

Because of the extreme polymorphism or variation of the HLA tissue typing system the chance of finding unrelated matches is approximately 1:100,000, necessitating the establishment of large suitably typed panels. This genetic variation is particularly problematic where there exists, additionally, a diversity of populations, including
many of African ancestry that carry with them rare antigens thereby emphasising dependence on international collaboration on the one hand and expanding local donor base on the other.

The main aim of the SABMR is to recruit, maintain and expand availability suitable volunteers for South African patients requiring haematopoietic stem cell transplant. In parallel a priority is to continue conducting and promoting relevant research into this system among the indigenous people of sub-Saharan Africa with their unique genetic constitution seeking to identify new antigens and so contribute to both knowledge and provision of matched unrelated grafts.

The South African Lymphoma Study Group

Peter Jacobs¹, Erna Mansveldt² and Colleen Wright³

¹Haematology Department, Constantiaberg Medi-Clinic, Cape Town
²Haematological, ³Anatomical Pathology, University of Stellenbosch and Tygerberg Hospital

20 years ago Professor Peter Jacobs, from the University of Cape Town, Professor Geoffrey Falkson from the University of Pretoria and Professor Werner Bezwooda of the University of Witwatersrand, supported by Bristol Meyers Squibb with Mrs. Esmé Goodwin as the secretary, established a meeting to be held every second year. The focus was to be on the continued understanding of the clinical haematology and haematopathology of these tumours as a basis for relevant local research on the one hand and dissemination of knowledge and maintenance of internationally acceptable treatment programmes on the other. The intention was that an independent and non-parochial governance would provide an interactive forum for the discussion of Hodgkin’s disease and the remaining lymphomas. To encourage the balance between clinical and laboratory-based studies, at an educational as well as scientific level, the forum was designed around a series of plenary presentations whilst, at the same time, strongly encouraging the presentation of local South African investigations. The importance of paramedical professionals was recognised and holding the congresses at suitable venues without registration fee encouraged the participation of nurses, pharmacists and laboratory technologists. The role of the pharmaceutical industry has been acknowledged and commercial exhibits remain an integral part of the two or three day symposia.

A particularly successful meeting was held in 2000 with a strong local faculty namely Dr. Philip Barlow Mills, Dr. Jack Bergman, Dr. Ellen Bolding, Dr. David Eedes, Professor Arderne Forder and distinguished international authorities including Dr. James O Armitage from Omaha Nebraska, Professor Borje Andersson and Professor Noel Buskard.

The 10th biennial will take place at the Constantiaberg Medi-Clinic in October 2002. The raison d’être will again be to provide broad interaction between clinical and haematopathologic aspects of these challenging groups of lymphoreticular tumours. With rapid progress in the field the particular discussion points will include the utility of fine needle aspiration linking flow cytometry to conventional cytology and immunohistochemistry on the one hand with
KEYNOTE ADDRESS

Bone marrow transplantation: how important is CD34 cell dose in HLA-identical stem cell transplantation?

S. Heimfeld

Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

A recent analysis of Fred Hutchinson Cancer Research Center data has been undertaken to investigate the association of infused CD34 cell dose with various clinical outcomes after HLA-identical transplantation. Separate assessments for unrelated vs related donors and the use of bone marrow or mobilized G-CSF-primed peripheral blood mononuclear cells (G-PBMC) have been incorporated. The three primary findings are: (1) Higher CD34 dose results in better neutrophil and platelet recovery in all settings. (2) Higher CD34 doses (>8 x 10^6/kg) are associated with the development of more chronic graft-versus-host disease when using related G-PBMC. (3) Higher CD34 dose is correlated with improved survival after unrelated donor bone marrow transplantation. These data suggest that the CD34 content of a graft can have a significant impact on clinical outcome after allogeneic transplantation, but defining an optimal dose is dependent on both the type of donor and the stem cell source.

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Keywords: allogeneic transplantation; CD34 dose; engraftment; GVHD; survival

Introduction

After allogeneic stem cell transplantation, outcomes such as survival or graft-versus-host disease (GVHD) depend on many patient-specific factors, but are also influenced by the source of stem cells, the make-up of different cell populations within the graft, and the degree of HLA matching between the donor and the recipient.1-3 This is in contrast to the Fred Hutchinson Cancer Research Center's (FHRC) experience with both matched related and related unrelated donors, the use of bone marrow or G-CSF mobilized peripheral blood mononuclear cells (G-PBMC) grafts, and quantitative enumeration of CD34+ hematopoietic stem/progenitor cells in those grafts is correlated with various clinical outcomes. One of the relevant questions that can be addressed with our large database of patient transplants is whether outcomes such as engraftment, GVHD and survival are associated with the number of CD34+ cells infused into the recipient. Data on the association of cell dose with outcome after cord blood transplantation has been recently published, and will not be covered in this paper.4-6

One of the first considerations in this type of analysis are other variables such as the type of donor, related vs unrelated, and the source of CD34+ cells, bone marrow vs G-PBMC. These factors have clearly shown to have an influence on clinical outcomes, and thus an analysis of CD34+ cell dosage effects must take these variables into account. Similarly, different clinical endpoints such as engraftment, GVHD, or survival must also be analyzed separately, as there is no a priori reason that particular CD34+ cell doses will yield identical results in various clinical settings. Finally, statistical methods must be employed to ensure that other patient parameters (age, type of disease) that can also affect these clinical outcomes are accounted for in these correlative studies.

Quantitative flow cytometry is the preferred method to determine the number of CD34+ cells in a bone marrow or G-PBMC graft. There are a variety of different techniques that are employed among clinical laboratories, including the ISHAGE, Milan, and Norway protocols. Each of these methods utilizes slightly different staining combinations and gating strategies that can result in somewhat different values. Thus, when reporting CD34 dose-response relationship, the actual technique used must be acknowledged as that may have an effect on the reported values. It is important to keep in mind that when a specific dose is referred to here in this paper, it may not be exactly the same dose that would be determined by another lab using a different procedure. At the FHRC, we routinely use a three-color combination of CD34-PE/CD14-FITC/7-AAD staining. Samples are first gated on total nucleated cells, followed by exclusion of nonviable 7-AAD+ cells, followed by exclusion of CD14+ high-side scatter populations (previous work has shown that no true CD34+ cells are also CD14+), followed by gating on the brightest expressing CD34+ cell population.66 It is this type of flow cytometry analysis that has been used for the correlative studies reported in this paper.

The FHRC has developed a fairly large database over the last few years in which CD34 dosage information in the grafts has been determined. With respect to the different patient subgroups, there are currently 171 related and 265 unrelated bone marrow donors, while there are 371 related and 75 unrelated G-PBMC donors. The median doses of CD34+ cells vary between the subgroups, for bone marrow donors, related vs unrelated, the medians are 3.0 x 10^6/kg (range 0.8-19) and 2.8 x 10^6/kg (range 0.3-26). For G-PBMC donors, related vs unrelated, the medians are much higher, 8.5 x 10^6/kg (range 1-34) and 8.1 x 10^6/kg (range 1.2-34). Thus, comparing bone marrow vs G-PBMC, the doses of CD34+ cells differ on average by approximately three-fold, which could have a potentially important impact on clinical outcomes.

Engraftment

In terms of short-term neutrophil engraftment, there is a significant difference between G-PBMC and bone marrow even when comparing similar doses of CD34 cells, with G-PBMC generally showing 5-7 days faster recovery times. Within the related G-PBMC subgroup, days to recovery of absolute neutrophil counts (ANC) greater than 500/μl plotted against infused CD34+ cell dose does show an association, higher dose
yielding shorter times to engraftment. However, whether this is a clinically significant effect is somewhat up for debate. There does not appear to be a strict dose–response relation, with at most a 1–2 day maximum difference in terms of ANC recovery between the lower and higher CD34 doses. This may partly be an artifact of clinical trial design. Nearly all FHRC allogeneic protocols require a minimum cell dose of 4 x 10^6 CD34+ cells/kg. Thus, there are relatively few patients below this level, which may be above the threshold value where more significant delays in ANC recovery would occur.

The story with respect to platelet engraftment is very similar. Again there is a statistically significant association with higher CD34 dose yielding faster recovery times, with G-PBMC showing 5–12 days faster engraftment than bone marrow. At lower CD34 doses with related G-PBMC (<6 x 10^6/kg actual patient body weight), 10% of the patients do not recover their platelet counts within 30 days after transplantation, whereas at higher dose ranges (>10 x 10^6/kg), all patients recover their counts above 20 000 by day 20. A similar effect is seen with slightly different kinetics for related bone marrow, where, in this case, patients with the lowest doses (<2 x 10^6/kg) show a longer time for platelet engraftment and a much higher proportion of patients do not recover their platelet counts to 20 000 before day 30.

Chronic extensive GVHD

In the related bone marrow setting, CD34 dose does not appear to correlate with chronic extensive GVHD (cGVHD), and with unrelated G-PBMC, we do not have enough data and long-term follow-up to make any definitive conclusions. With unrelated bone marrow transplantation there is a suggestion that higher CD34 doses do lead to increases in cGVHD, although this has not reached statistical significance. Our best correlation of CD34 dose and cGVHD has been obtained with related G-PBMC. We have previously published that, comparing patients that receive CD34 doses greater than 8 x 10^6/kg vs all others, there is a significant increase in cGVHD from 40 to 50%. We have further explored this association in the current database. Separating the range of CD34 dosages into 20% quintiles, the lower four quintiles show identical levels of cGVHD, while the 5th quintile (>10 x 10^6/kg) again shows a significantly higher rate of cGVHD. The biological explanation for this finding is unclear, one possible hypothesis for increased cGVHD may have to do with better reconstitution of donor dendritic cells from the larger number of CD34+ cells within the graft.12

Overall survival

Within the FHRC data there appears to be no relationship between CD34 doses and overall survival in the case of related bone marrow or related G-PBMC. For related bone marrow, this finding is different from what has been published from other institutions. However, in the case of unrelated G-PBMC there is a suggestion for CD34 dose affecting survival outcomes. Separating the dose of CD34 into quintiles, the two lowest quintiles (<7 x 10^6/kg) show the worst outcomes with survival at 1 year of 30–40%. The third and the fourth quintiles (7–10 x 10^6/kg) do better, with 1-year survival at 60–70%. The fifth quintile (>10 x 10^6/kg) starts out similar to the third and fourth, but then tends to drop off with time so that at 1-year survival was only 40%. This sort of effect may indicate that, in the unrelated G-PBMC setting, an optimum CD34 dose may lie somewhere in the middle. Lower CD34+ cell dose is not optimal, and the highest CD34 doses are also not good, but this may be for a different reason such as increased GVHD (see above). Additional data, further follow-up, and more detailed analyses will be required to better explore this finding.

The most significant association of CD34 dose with survival was observed with the unrelated bone marrow transplants. Again splitting the CD34 dose into quintiles, a clear dose–response relation was observed, with the lowest quintile having 40% survival at 2 years, next quintile at 50%, and the other quintiles at 60–70%. A more extensive multivariate analysis of this data adjusting for patient age and disease risk indicated that
the most significant predictor for overall mortality was the dose of CD34+ cells. This is shown graphically in Figure 1, where the unrelated bone marrow patients have been split into two groups, above and below the median CD34 dose of 2.7 x 10^6/kg. The improvement in overall survival seems to be driven by a reduction in nonrelapse mortality, that is, no difference in the relapse rates was seen between the patients getting the lower vs higher CD34 doses. There was a suggestion of an association with grades 3 and 4 acute GVHD, again with higher doses giving a lower effect. There was no evidence for any effect on chronic GVHD. In this analysis we also looked at a variety of other cell populations: CD14 monocytes, T-cell subset, B-cells, and NK-cells, but these other cell types did not change or improve the model to any significant extent. The best and only cell population that significantly predicted for improved survival was the dose of CD34+ cells.\textsuperscript{15}

Conclusion

A prospective randomized trial has shown that there is a survival advantage for matched related allogeneic transplant recipients who received G-PBMC vs those who received bone marrow as a source of stem cells.\textsuperscript{14} The biological basis for this advantage is not entirely clear. As noted above, G-PBMC have on average three-fold more CD34+ cells than bone marrow. However, this is unlikely to be the whole explanation for the improved survival, in almost every other lineage such as T-cell subsets, monocytes, B-cell, and NK cells, the differences are even larger (10-30-fold; see Figure 2). The association of these other cell populations with clinical outcomes is just beginning to be investigated. In a preliminary analysis for correlation with overall survival, higher doses of NK cells and particularly the TNK type cells seem to be the best predictors for improved survival after matched related allogeneic G-PBMC transplantation. In addition, there can also be qualitative as well as these quantitative differences. G-CSF mobilization and apheresis detection does result in significant changes in gene expression within the CD34 compartment when compared with bone marrow.\textsuperscript{15} Other changes also occur in dendritic cell subsets after G-CSF mobilization.\textsuperscript{16}

In conclusion, we have examined the dose–response relationship of CD34+ cells in related and unrelated donor bone marrow or G-PBMC in terms of clinical outcomes such as engraftment, chronic GVHD and overall survival. In all cases, we can see that higher CD34+ cell doses lead to better recovery of neutrophils and platelets, although the clinical significance of that improved recovery is debatable. With related bone marrow there is no association of CD34+ dose with chronic GVHD or overall survival, but with unrelated bone marrow, higher CD34 dose does result in improved survival. In the related G-PBMC setting, no significant association is seen with overall survival with our present data; however, higher CD34+ doses (> 10 x 10^6/kg) are associated with more chronic GVHD. With unrelated G-PBMC, there is a suggestion that higher CD34 dose is associated with better survival, but more data and more long-term follow-up are necessary before a definite conclusion can be made. Finally, we are continuing to examine the association of other cell populations with clinical outcomes in each of these different clinical settings.

Acknowledgements

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References

Allogeneic Peripheral Blood Stem-Cell Compared With Bone Marrow Transplantation in the Management of Hematologic Malignancies: An Individual Patient Data Meta-Analysis of Nine Randomized Trials

Stem Cell Trialists' Collaborative Group

ABSTRACT

Purpose
Considerable uncertainty exists regarding relative effects of allogeneic peripheral blood stem cells transplantation (PBSCT) versus bone marrow transplantation (BMT) on outcomes of patients with hematologic malignancies.

Patients and Methods
To provide the totality of research evidence related to the effects of PBSCT versus BMT, we conducted an individual-patient data meta-analysis using data from nine randomized trials enrolling 1,111 adult patients.

Results
Compared with BMT, PBSCT led to faster neutrophil (odds ratio [OR] = 0.31; 95% CI, 0.25 to 0.38; P < .00001) and platelet engraftment (OR = 0.52; 95% CI, 0.44 to 0.61; P < .00001). PBSCT was associated with a significant increase in the development of grade 3-4 acute graft-versus-host disease (GVHD; OR = 1.39; 95% CI, 1.03 to 1.88) and extensive (47% v 31% at 3 years; OR = 1.89; 95% CI, 1.47 to 2.42; P < .000001) and overall chronic GVHD (68% v 52% at 3 years; OR = 1.92; 95% CI, 1.47 to 2.49; P < .000001), but not grade 2-4 acute GVHD (54% v 53%; P = .49). PBSCT was associated with a decrease in relapse (21% v 27% at 3 years; OR = 0.71; 95% CI, 0.54 to 0.93; P = .01) in both late-stage (33% v 51% at 3 years; OR = 0.59; 95% CI, 0.38 to 0.83; P = .02) and early-stage disease patients (16% v 20% at 3 years; OR = 0.69; 95% CI, 0.49 to 0.98; P = .04). Norelapse mortality was not different between groups. Overall and disease-free survival were only statistically significantly improved in patients with late-stage disease (overall survival: 46% v 31% at 3 years; OR = 0.64; 95% CI, 0.46 to 0.90; P = .01; disease-free survival: 41% v 27% at 3 years; OR = 0.63; 95% CI, 0.45 to 0.87; P = .01).

Conclusion
PBSCT is associated with a decreased relapse rate in hematologic malignancies and improvement in overall and disease-free survival in patients with late-stage disease. PBSCT is also associated with a significant risk of extensive chronic GVHD.

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While peripheral blood stem cells (PBSC) are used almost exclusively in autologous transplantation, recent surveys indicate that PBSC are used in 50% to 60% of allogeneic stem-cell transplants. Thus, large variation in practice and considerable uncertainty exists with respect to the relative effects of allogeneic PBSC transplantation (PBSCT) versus bone marrow transplantation (BMT) on the outcomes of patients with hematologic malignancies. In order to address this question, several randomized controlled
PBSCT v BMT for Hematologic Malignancies

trials have been conducted. Despite several well designed and executed clinical trials, taken individually, most of these trials were too small to draw definitive conclusions, and not surprisingly, substantial controversy still remains regarding the impact on the occurrence of graft-versus-host disease (GVHD), mortality, disease control, and other important clinical outcomes.

This controversy is typical in health care research and demonstrates the need for a systematic review to assemble the totality of relevant research evidence to determine the relative merits of new interventions and therapies. The "gold-standard" for combining evidence from existing randomized trials is an individual patient data meta-analysis (IPD-MA) in which updated data on each and every participant from each and every relevant trial are centrally collected, processed, and analyzed. Here, we report the first IPD-MA examining the differences in the outcomes between human leukocyte antigen (HLA)–matched, related allogeneic PBSCT and BMT as therapy for hematologic malignancies.

Recommended procedures for the meta-analysis based on the individual patient data were followed. Randomized controlled trials (RCTs) in which adult patients with hematologic malignancies and HLA-matched sibling donors were randomly assigned to PBSCT and BMT were eligible for the analysis. We performed an extensive search of a number of computerized databases (MEDLINE, EMBASE, LILACS, CANCERLIT, The Cochrane Library) and the abstracts of meetings of the American Society of Hematology, European Hematology Association, American Society of Clinical Oncology, IBMT (International Bone Marrow Transplant Registry), and EBMT (European Group for Blood and Marrow Transplantation) from 1990 to 2002. Experts in oncology and hematology were asked about ongoing or closed studies that had not yet been published. Details of the search strategy were published as a Cochrane protocol. Periodic searches were subsequently performed with the cutoff for the trial identification and data collection as of August 2003. Once eligible trials were identified, their principal investigators were contacted, and a central database was formed. Demographic data (patient and donor age and sex, diagnosis and disease status at the time of transplantation, cytomegalovirus serology); information regarding the transplantation procedure (date of random assignment and of transplantation, allocated treatment [PBSCT or BMT], conditioning regimen used [total-body irradiation–based v non–total-body irradiation–based], graft processing and manipulation, number of CD3 cells/kg and C34 cells/kg transplanted, GVHD prophylaxis, use of post-transplantation growth factors); and details of the trial design, including randomization methodology, were collected. The following outcome data were collected for each patient: time to neutrophil and platelet engraftment, date of relapse or disease progression, date of onset and grade of acute GVHD and chronic GVHD, and the date of last follow-up or death. Cause of death was distinguished between relapse-related and non–relapse-related.

Extensive data checking was performed using the methods described previously. Data were checked for obvious inconsistencies and were amended as necessary through intensive correspondence with the responsible principal investigators. Raw data were also compared with aggregate data in available publications. Detailed checks for any imbalance in accrual between two randomized arms, follow-up and length of follow-ups, and the numbers in subgroups were also performed.

All comparisons were based on an intention-to-treat principle. Individual patient data allowed calculation of required statistics using the exact dates of events, which is more statistically reliable and clinically informative than basing the calculations on proportions alive at a particular point in time. Briefly, the number of events observed (O) in the PBSCT arm of each trial is compared with the number expected (E) if the events in that trial had been equally distributed between the PBSCT and BMT arms. The difference between these numbers, O - E, and its variance yields the log-rank test for each trial. The sum of the statistics is first produced for each trial. The individual log-rank statistics from each trial were then combined to give an overall estimate of the effect of PBSCT versus BMT on the outcomes of interest. The important point here is that the analysis is not done by pooling all patients in one mega-analysis, but rather pooling was done by combining individual log-rank statistics to obtain the overall log-rank statistics for all trials, which are then used to calculate reductions of overall odds of death or other outcomes of interest. This means that the methods employed in our analysis preserve the original randomization in each trial since they do not involve any analyses in which patients in one trial are directly compared with patients in another trial. The results are expressed in such a way that the annual odds of event of interest of 0.75 might equivalently be described as an odds ratio, a hazard ratio of 0.75, an odds reduction of 25%, or a 25% reduction in the event rate. Assumption-free methods were used. All P values are two-tailed.

Heterogeneity (ie, variability or differences between studies in the estimates of effect) was also assessed. We evaluated methodological heterogeneity (differences in study design), clinical heterogeneity (differences between studies in key characteristics of the participants, interventions, or outcome measures; Table 1), and statistical heterogeneity. Formal tests for heterogeneity were performed to investigate whether the effect size might be different among the studies/subgroups (ie, if observed variability in results is greater than that expected to occur by chance). All subgroup analyses were defined a priori.

The main endpoints analyzed were: overall survival, relapse/progression, GVHD, disease-free survival, death in remission and engraftment. Time was calculated from the date of randomization; in the case of acute and chronic GVHD it was calculated from the date of randomization, date of transplant, and day + 100 after the transplantation (in case of chronic GVHD). Since the results did not change appreciably, only the latter analyses are shown. Disease-free survival was defined as time to death or relapse, whichever occurred first. Due to small numbers, the analyses according to disease types were not reliable and were, therefore, supplemented with the analysis according to disease prognostic features. A uniform consensus among all trialists was achieved to separate patients into those with "early-stage" (chronic myelogenous leukemia [CML] in first chronic phase, acute myeloid leukemia [AML] and acute lymphoblastic leukemia [ALL] in first complete remission, and refractory anemia/refractory anemia with ringed sideroblasts subtypes of myelodysplastic syndromes [MDS]) and "late-stage" disease (CML in second chronic phase, accelerated phase or blast crisis AML or ALL, refractory or in...
Table 1. General Characteristics of the Randomized Clinical Trials That Compared PB SCT Versus BMT for the Treatment of Hematologic Malignancies.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Comparison</th>
<th>Eligibility Criteria</th>
<th>Setting</th>
<th>G-CSF Dose Employed for PBSC Mobilization</th>
<th>Routine G-CSF Posttransplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>G-PB SCT versus G-BMT</td>
<td>HLA-identical sibling donors</td>
<td>Single-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>No</td>
</tr>
<tr>
<td>Brazil</td>
<td>PBSC versus BMT</td>
<td>Patients 10-60 years old HLA-identical sibling donors</td>
<td>Single-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>No</td>
</tr>
<tr>
<td>Canada</td>
<td>PBSC versus BMT</td>
<td>Patients 16-65 years old CML (in chronic or accelerate phase)</td>
<td>Multi-center</td>
<td>5 µg/kg/d (Filgrastim)</td>
<td>No</td>
</tr>
<tr>
<td>EBMT/Amon</td>
<td>PBSC versus BMT</td>
<td>Patients 16-55 years old De novo AML, and ALL (in 1st or 2 nd remission or in 1st incipient relapse), CML in chronic or tolerate phase, MDS, AML (M3) (RAEB1) HLA-identical sibling donors</td>
<td>Multi-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>Yes</td>
</tr>
<tr>
<td>Egypt</td>
<td>PBSC versus BMT</td>
<td>Patients 18-62 years old HLA-identical sibling donors</td>
<td>Single-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>Yes</td>
</tr>
<tr>
<td>France</td>
<td>PBSC versus BMT</td>
<td>Patients &lt; 55 years old HLA-identical sibling donors</td>
<td>Multi-center</td>
<td>10 µg/kg/d (Lenograstim)</td>
<td>No</td>
</tr>
<tr>
<td>Netherlands</td>
<td>T-cell depleted (TCI) PB SCT versus TCI BMT</td>
<td>Patients 16-60 years old AML, ALL, MDS, MM, NHL</td>
<td>Multi-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>No</td>
</tr>
<tr>
<td>Norway</td>
<td>PBSC versus BMT</td>
<td>Patients 16-60 years old AML, ALL, CML, PM and MDS</td>
<td>Single-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>No</td>
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<tr>
<td>South Arabia</td>
<td>PBSC versus BMT</td>
<td>HLA-identical sibling donors</td>
<td>No</td>
<td></td>
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<tr>
<td>UK</td>
<td>PBSC versus BMT</td>
<td>Patients 15-55 years old HLA-identical sibling donors</td>
<td>Multi-center</td>
<td>10 µg/kg/d (Lenograstim)</td>
<td>No</td>
</tr>
<tr>
<td>US3</td>
<td>PBSC versus BMT</td>
<td>Patients 12-65 years old HLA-identical sibling donors</td>
<td>Multi-center</td>
<td>10 µg/kg/d (Filgrastim)</td>
<td>No</td>
</tr>
<tr>
<td>US2</td>
<td>PBSC versus BMT</td>
<td>CML HLA-identical sibling donors</td>
<td>No</td>
<td></td>
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</tr>
</tbody>
</table>

Abbreviations: GVHD, graft versus host disease; G-CSF, granulocyte colony-stimulating factor; BMT, bone marrow transplantation; PB SCT, peripheral blood stem cell transplantation; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML; chronic myeloid leukemia; MDS, myelodysplastic syndrome; PMF, primary myelofibrosis; NHL, non-Hodgkin's lymphoma; RAEBT, refractory anemia with excess blasts in transformation; TBI, total body irradiation; Bu, busulfan; Cs, cyclophosphamide; CSA, cyclosporine A; MTX, methotrexate; Pred, prednisone; EBMT, European Group for Blood and Marrow Transplantation.

Trials and Patients

We identified 12 RCTs enrolling 1,318 patients with various hematologic malignancies that compared HLA-matched, related allogeneic PB SCT with BMT (Sahovic E et al, "Allogeneic peripheral blood vs. bone marrow transplant in hematologic malignancies: A randomized trial,"
Table 1. General Characteristics of the Randomized Clinical Trials That Compared PBSC Versus BMT for the Treatment of Hematologic Malignancies (continued)

<table>
<thead>
<tr>
<th>Patients Characteristics</th>
<th>Conditioning Regimen</th>
<th>Notable Outcomes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GVHD Prophylaxis*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSAMTX (ID:1,3,6)</td>
<td>45</td>
<td>15-80 27 48.2</td>
</tr>
<tr>
<td>CSAMTX (ID:1.3,6,11) or CSAPRED (3 patients in BMT arm)</td>
<td>31</td>
<td>7-60 38 67.8</td>
</tr>
<tr>
<td>CSAMTX (ID:1,3,6,11)</td>
<td>46</td>
<td>19-65 133 58.3</td>
</tr>
<tr>
<td>CSA/MTX (ID:1,3,6)</td>
<td>38</td>
<td>17-88 196 56.0</td>
</tr>
<tr>
<td>CSA/MTX (ID:1,3,6,11)</td>
<td>22</td>
<td>16-42 23 76.6</td>
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<tr>
<td>CSA/MTX (ID:1,3,6,11)</td>
<td>36</td>
<td>16-63 53 52.5</td>
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<tr>
<td>CSA/MTX (ID:1,3,6,11)</td>
<td>41</td>
<td>18-60 76 63.3</td>
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<tr>
<td>CSA/MTX (ID:1,3,6,11)</td>
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<td>15-55 38 82.3</td>
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<td>CSAMTX (ID:1,3,6,11)</td>
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<td>15-48 45 54.2</td>
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<td>22-52 29 74.3</td>
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<td>CSAMTX (ID:1,3,6,11)</td>
<td>46</td>
<td>12-66 122 63.3</td>
</tr>
<tr>
<td>CSAMTX (ID:1,3,6,11)</td>
<td>46</td>
<td>12-66 122 63.3</td>
</tr>
</tbody>
</table>

11 of which have been published at the time of the analysis (Table 1). The requirements of the QUOROM (Quality of Reports of Meta-analyses of Randomized Controlled Trials) statement were followed.44 Individual patient data were provided on 1,288 patients from 11 trials (Table 1).

Table 1 presents the characteristics of all trials and patients considered in our analysis. Two trials differed substantially in the design from the rest: the Dutch trial included T-cell depletion of the grafts, while the Australian trial examined PBSC versus granulocyte colony-stimulating factor primed BMT. It was unanimously agreed by all trialists that these trials should not be included in the pooled analysis. While some differences existed among the other trials, it was felt that all trials tested similar interventions for similar conditions under similar circumstances to allow pooling of their data in this meta-analysis.6 Data from one trial (N = 29) were not provided, but were extracted from the published report of this trial when possible.18 Inclusion or exclusion of the data from this trial did not materially alter the results. Thus, data on 1,111 patients from nine trials were included in the final analysis. Overall, treatment groups appeared well balanced according to the most important prognostic features (i.e., age, sex, disease type, and so on; Table 1). Figure 1 shows the patient distribution according to the prognostic features. Median duration of follow-up of patients included in our analysis was 2.7 years (range, 0 to 8.6 years).

The main results of the analysis are summarized in the forest plot shown in Figure 2.

**Engraftment**

There was a highly significant reduction in the number of days to reach the absolute neutrophil count of 0.5 × 10^9/L in the patients receiving PBSC, and a platelet count greater than 20 × 10^9/L. Median time to neutrophil and platelet engraftment was 14 ± 21 days (X^2 = 97.59; P < .0001) and 14 ± 22 days (X^2 = 53.3; P < .0001) in the PBSC and BMT arms, respectively.

**Acute GVHD**

There was no difference in overall incidence in acute GVHD grades 1-4 between the two treatment groups (54%
v 53%; P = .49). Forty percent of the patients developed grade 2-4 acute GVHD with no significant increase in the rate after PBSCT (odds ratio [OR] = 1.14; 95% CI, 0.93 to 1.4; P = .2; Fig 3A). However, grades 3-4 acute GVHD occurred more often after PBSCT (by approximately 6% at 100 days [χ² = 4.58; P = .03]; OR = 1.39 [95% CI, 1.03 to 1.88]; Fig 3B).

**Chronic GVHD**

There was a highly significant increase in the odds of developing both extensive stage (OR = 1.89; 95% CI, 1.47 to 2.42; P < .000001) and overall (any stage; OR = 1.92; 95% CI, 1.47 to 2.49; P < .000001) chronic GVHD in patients treated with PBSCT. At 3 years, 47% and 68% of patients treated with PBSCT developed extensive or any stage chronic GVHD versus 31% and 52%, after BMT, respectively (Figs 4A and B). At 5 years, the corresponding figures for PBSCT versus BMT were 51% v 35% for extensive stage chronic GVHD and 73% v 56% for any stage, respectively. The results remained virtually the same regardless of the start date of calculation of chronic GVHD (ie, whether it was from the date of randomization, date of transplant or day + 100 after transplantation, respectively).

**Relapse, Relapse Mortality, and Nonrelapse Mortality**

Relapsed or progression rate at 3 and 5 years in PBSCT arm was 21% and 24% v 27% and 32% in the patients treated with BMT, respectively (OR = 0.71; 95% CI, 0.54 to 0.93; P = .01; Fig 5). The difference in the early-disease group was 16% v 20% at 3 years, and 16% v 25% at 5 years, respectively (OR = 0.69; 95% CI, 0.49 to 0.98; P = .04). In the late-disease group, the difference was 33% v 51% at 3 years and 44% v 58% at 5 years, respectively (OR = 0.59; 95% CI, 0.38 to 0.93; P = .02; Fig 6), but the test for interaction between the two groups was not significant (P = .6).

Eleven percent and 14% of patients died due to relapse/progression of disease in the PBSCT group, versus 16% and 18% in the BMT arm at 3 and 5 years, respectively (χ² = 5.51; P = .02). Relapse-related mortality followed the same pattern, indicating that once disease relapsed/progressed, a salvage treatment may, on average, not be effective (Fig 7A).
Approximately 30% of patients died in both groups due to nonrelapse causes (OR = 0.99; 95% CI, 0.79 to 1.25; P = 1.0). As expected, most deaths due to treatment occurred early after transplantation (Fig 7B).

**Disease-Free Survival**

Overall, allogeneic PBSC was associated with a statistically significant improvement in disease-free survival over BMT (OR = 0.80; 95% CI, 0.67 to 0.97; P = .02; 59% vs 53%
at 3 years and 54% vs 47% at 5 years, respectively; Fig 8). This difference was more pronounced in patients with late disease (41% vs 27% at 3 years; and 32% vs 21% at 5 years, respectively; OR = 0.63 95% CI, 0.45 to 0.87; \( P = .01 \)) than in patients with early disease (OR = 0.85; 95% CI, 0.67 to 1.08); \( P = .2 \); Fig 9). However, a test for interaction between the two groups was not significant (\( P = .1 \)).

### Survival

There was no statistically significant difference in overall survival between PBSC and BMT (OR = 0.87; 95% CI, 0.72 to 1.06; \( P = .17 \); Figs 10 and 11). However, PBSC was associated with a higher 5-year overall survival probability in the subpopulation with late disease (46% vs 31% at 3 years, and 39% vs 29% at 5 years, respectively; OR = 0.64; 95% CI, 0.46 to 0.90; \( P = .01 \)) but not in patients with early disease (65% vs 64%; OR = 0.97; 95% CI, 0.75 to 1.25; \( P = .8 \); Fig 12). Test for interaction was borderline statistically significant (\( P = .05 \)).

Statistical heterogeneity of treatment effect between trials was noted only for relapse outcome (\( \chi^2 = 14.6; P = .04 \)) and neutrophil (\( \chi^2 = 32.2; P = .000008 \)) and platelet engraftment (\( \chi^2 = 33.6; P = .00005 \)).

### Subgroup Analyses

To elucidate if the effect of the type of transplantation differed among different disease groups, we performed a number of subgroup analyses. Briefly, the most consistent effect is seen in CML, in which PBSC was associated with improvement in relapse in all patients (OR = 0.34; 95% CI, 0.2 to 0.58), and improvement in disease-free and overall survival in patients with late-stage disease (OR = 0.28 [95% CI, 0.11 to 0.73] for disease-free survival, and OR = 0.31 [95% CI, 0.12 to 0.80] for overall survival, respectively). PBSC also led to improvement in disease-free survival (OR = 0.39; 95% CI, 0.21 to 0.72) and overall survival (OR = 0.45; 95% CI, 0.24 to 0.85) in AML patients with late-stage disease. Due to small number of patients (Fig 1), we could not confirm or refute superiority of PBSC versus BMT in other disease categories.

According to international registry data, approximately half of transplant physicians prefer allogeneic PBSC over BMT. To address the question of which source of hematopoietic stem cells might be preferable, we...
conducted the first IPD-MA of prospective randomized trials comparing transplantation of HLA-matched, related allogeneic PBSC and BMT in patients with hematologic malignancies. It is important to realize that our results and conclusions apply only to matched sibling myeloablative allogeneic transplantation and not to the role of nonmyeloablative transplantation or alternative donor strategies.

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**Fig 6.** Time-to-event plots showing the risk of relapse in patients with hematologic malignancies treated with allogeneic peripheral blood stem-cell transplantation (PBSC) versus bone marrow transplantation (BMT). (A) In early-stage disease and (B) in late-stage disease. Abs diff, absolute difference.

**Fig 2.** (A) Relapse-related mortality. Time-to-event plots showing the absolute risk reductions in relapse mortality in patients with hematologic malignancies treated with allogeneic peripheral blood stem-cell transplantation (PBSC) versus bone marrow transplantation (BMT). (B) Time-to-event plots showing the absolute risk reductions in death without relapse (nonrelapse mortality) in patients with hematologic malignancies treated with allogeneic PBSC versus BMT. Abs diff, absolute difference.
Our analysis should be interpreted within the context of the extreme logistical difficulties associated with performing large randomized trials in allogeneic transplantation. Because hematologic diseases are rare and transplant numbers are relatively low even in large centers, most trials chose to enroll patients with a variety of hematologic malignancies. However, by pooling all existing data, we were able to increase the power of the analysis and provide the most definitive evidence to date.

Our analysis demonstrated that stem cell source is not statistically significant in its effect on overall survival. However, PBSC is associated with an increase in both survival and disease-free survival in advanced-stage disease. PBSC is also associated with significantly more rapid neutrophil and platelet engraftment, a reduced relapse rate and an increase in the risk of chronic GVHD.

Our results suggest that patients with late-stage disease benefit from PBSC rather than BMT. This finding was consistent for all important clinical end points (survival, disease-free survival, relapse). However, statistical tests for interaction did not provide clear evidence of a difference in treatment effect between early- and late-stage disease, and further data are needed to confirm or refute this finding.

In early-stage disease, PBSC was associated with a lower relapse rate but this did not result in statistically significant better overall or disease-free survival. This could, in part, be a consequence of a large number of patients with chronic phase CML, a disease in which relapse after allogeneic transplant does not immediately lead to death, or alternatively, a reflection of the fact that in early stage disease, nonrelapse mortality is higher compared to relapse-related death.

Although all the trials included in this analysis attempted to address a similar treatment question, they also differed in some details (Table 1). This could have introduced both clinical and statistical heterogeneity into the results. However, except for neutrophil and platelet engraftment, heterogeneity between trials was absent or minimal. Even though the degree of the effects on engraftment was heterogeneous, engraftment was uniformly much more rapid after PBSC than BMT in all trials. This result is biologically plausible. Therefore, the observed heterogeneity of treatment effect between trials can be best explained by random fluctuations in the effect size.

One of the main purposes of meta-analyses is to investigate if any bias or play of chance could have affected the results of the analysis. Only if the intervention’s effect is greater than that of any of potential biases can the results be considered credible and reliable. The transplant field has...
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Fig 10. Forest plot illustrating the effect of peripheral blood stem-cell transplantation (PBSCT) versus bone marrow transplantation (BMT) on overall survival. Note: inclusion of Egyptian trial (N = 29) from which data on individual patients were not available (data from this trial were extracted from the paper). Inclusion of this trial did not change the results significantly (see the main text for details). NS, not significant; OR, odds ratio; EBM, European Group for Blood and Marrow Transplantation; O = E, observed = expected; Var., variance; Redn., reduction; SD, standard deviation.

traditionally been plagued with selection biases and attrition bias (ie, large imbalance in dropouts between treatment arms). Therefore, paying close attention to the quality of random assignment, which aims to control for selection bias, and accounting for adequate follow-up, to assess for attrition bias is critical. One of the methodological advantages of individual-patient data meta-analysis is to allow assessment of raw data for the quality of random assignment, concealment of allocation, balance in follow-up, drop-outs, confirmation of internal consistency, and performance of intention-to-treat analyses.

We have performed extensive data checking, including sensitivity analysis within each trial. Our analyses indicated no obvious sources of bias and overall consistent data of high quality. However, we have not controlled for observer bias, which is inherent in the assessment of outcomes such as GVHD resulting in poor interobserver agreement. In addition, chance could have affected some of the results because of the large number analyses performed on a relatively small data set.

In the transplantation literature, some trials employ competing risk analysis to address incidence of outcomes such as chronic GVHD. Since the purpose of our study was to compare the effects of PBSCT versus BMT, competing risks analyses were not undertaken as they can be misleading when making treatment comparisons, and methods are not well developed, particularly for use in meta-analysis. Similarly, since the main question being addressed relates to the relative effect of treatments on outcomes, it was inappropriate to evaluate the effect of one outcome (eg, GVHD) on the other (eg, relapse or survival), as well as time-dependent factors.

When choosing between two treatment interventions, a practitioner has to consider both benefits and harms associated with each of the treatment alternatives. Based on
this study, PBSCT is associated with faster engraftment, a decrease in relapse and improved disease-free survival when compared with BMT in HLA-identical sibling transplantation for hematologic malignancies. These outcomes were particularly evident in patients with late-stage disease where improved survival was also seen.

Indeed, the largest effect of PBSCT in terms of improvement of disease-free survival and overall survival was seen in patients with late-stage AML and CML. Unfortunately, we could not collect data on cyto genetics to further delineate effect on specific prognostic categories in AML. With regard to treatment of CML, recently, the new standard—imatinib—has emerged as the initial treatment of choice for the management of this disease.23 Our study was not designed to address the issue of relative merits of imatinib versus allogeneic transplantation. Nevertheless, it does appear that the dramatic shift in the contemporary practice did occur in the sense that fewer patients in the "good" risk category (ie, in chronic phase) are being referred to transplantation. This has resulted in more late-stage CML patients currently undergoing transplantation. Our results show that once patients have progressed to accelerated phase or blast crisis, their disease-free survival and overall survival after allogeneic transplantation are markedly superior after PBSCT compared with BMT.

The incidence of acute GVHD was the same in both PBSCT and BMT although severity (grade 3-4) was greater in recipients of PBSCT. Since more severe GVHD is
associated with increased mortality, this very likely accounts for the failure of PBSC to have a beneficial effect on overall survival, despite a lower rate of relapses. This potentially negative effect of peripheral blood stem cells would likely have a greater impact on survival among patients with less advanced leukemias, where rates of relapse are lower.

PBSC was associated with the increased risk of both limited and extensive chronic GVHD. Extensive chronic GVHD can adversely affect quality of life.25-35 As well as survival,26,27 but none of the trials included in our meta-analysis collected analyzable data on quality of life. Physicians and patients should weigh the higher risk of disease recurrence with BMT against long-term consequences of chronic GVHD when deciding which stem cell source to use,36 since chronic GVHD may be an important marker of an active graft-versus-leukemia response and may have been responsible for the reduction in relapse rates and increase in disease-free survival seen with PBSC. Future research should try to delineate not only harmful effects of chronic GVHD but also its potentially beneficial effects, particularly the impact of milder clinical presentations of chronic GVHD on clinical outcomes in the different clinical subgroups. Ultimately, the choice of treatment should be discussed with the patient, with particular emphasis on these critical trade-offs.

Our analysis reiterates the limitations of small randomized studies.28 By synthesizing the totality of research evidence we were able to: (1) resolve apparent inconsistencies in clinical outcomes reported by individual trials and (2) identify conclusively important clinical effects that had not been uniformly demonstrated with the previously available information. However, long-term follow-up will still be necessary for fuller understanding of the role of the stem cell source on clinical outcomes since overall follow-up of patients included in our analysis was relatively short.

Appendix: Members of Stem Cell Trialists’ Collaborative Group (in alphabetical order):
1. Mahmoud al-Jurf—Department of Medicine, King Faisal Specialist Hospital & Research Center, Riyadh, Kingdom of Saudi Arabia
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3. Claudio Annasetti, H. Lee Moffitt Cancer Center & Research Institute, University of South Florida, Tampa, FL
4. Jane F. Apperley—EBMT—Department of Haematology, Faculty of Medicine, Imperial College, Hammersmith Hospital, London, UK
5. Roy Baynes—Amen, Thousand Oaks, CA
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8. M. Ashraf Chaudhary, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD
9. Mike Clarke—Clinical Trial Service Unit, Oxford University, UK
10. Jan J Cornelissen—Department of Hematology, Daniel den Hoed Cancer Center, Rotterdam, the Netherlands
11. Stephen Couban—Canadian Bone Marrow Transplant Group, Department of Medicine, Dalhousie University and Queen Elizabeth II Health Sciences Centre, Halifax, NS, Canada
12. Corey Cutler—Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA
13. Benjamin Djulbegovic—H. Lee Moffitt Cancer Center & Research Institute, University of South Florida, Tampa, FL
14. Martin Gyger, Hospital General Juif, McGill University, Canada
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16. Dag Heldal—Medical Department, Rikshospitalet University Hospital, 0027 Oslo, Norway
17. Robert K Hills University of Birmingham Clinical Trials Unit, University of Birmingham, Birmingham, UK
18. Bronno Van der Holt—Department of Trials & Statistics—HOVON Data Center Erasmus MC—Daniel den Hoed, Rotterdam, the Netherlands
19. Istok Hozo—Department of Mathematics, Indiana University, Gary, IN
20. Mathieu Kuentz, MD, Department of Hematology, Hospital Henri Mondor, Creteil and French society of transplantation (SFGM-TC), France
21. Ambuj Kumar—H. Lee Moffitt Cancer Center & Research Institute, University of South Florida, Tampa, FL
22. Jeff Lipton, Princess Margaret Hospital, University Health, Network in Toronto Faculty of Medicine at the University of Toronto, Canada
23. James Matcham, Aegen, Thousand Oaks, CA
24. Mohammad Mohty—France—Unité de Transplantation et de Therapie Cellulaire (UTTC), Institut Paoli-Calmettes, Marseille and French society of transplantation (SFGM-TC), France
25. James Morton—Bone Marrow Transplant Unit, Royal Brisbane Hospital, Herston, Australia
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27. Ray Powles—Royal Marsden Hospital, Sutton, UK
28. Sue M. Richards, Clinical Trial Service Unit, Oxford University, UK

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Authors’ Disclosures of Potential Conflicts of Interest

Although all authors have completed the disclosure declaration, the following authors or their immediate family members have indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO’s conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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7. Stewart L, Clark M: For the Cochrane Collaboration Working Group on meta-analyses using individual patient data: Practical methodology of meta-analyses (overview) using up-...
way to encourage young doctors, specialists or generalists, to work in the places where they are really most needed, namely in the country districts. Is it any wonder that young doctors are leaving this country to practise elsewhere?

Justin van Selma
PO Box 125
Pietersburg 5000

BONE MARROW TRANSPLANTATION IN THE PRIVATE SECTOR

To the Editor: To place differing viewpoints about bone marrow transplantation in perspective, we present an analysis of the first 209 consecutive patients grafted at our unit from 1 January 1995. All data are audited by the International, Autologous and European Bone Marrow Transplantation Registries as a requirement for continued accreditation.

Our current protocol is an extension of that used in the first South African transplant, carried out in December 1972. The increasing application and principles of patient selection and technique have recently been summarised.

A fundamental difference exists between this approach to autologous grafting and other methods. Thus, T-cell depletion as the sole form of immunosuppression is achieved by exposure to a specific monoclonal antibody in the-bag. Engraftment was universal, relapse rates were low and, most notably, graft-versus-host disease was not seen. A further modification has been the discontinuation of bone marrow as the source of haematopoietic stem cells and its replacement by the corresponding immunohaematopoietic population harvested from the peripheral blood. In addition it has been demonstrated that the newly engineered human variant of the antibody designated 1H and 1G is equally effective.

Outcome depends on operation of an optimal physical facility staffed by a motivated multidisciplinary team and incorporating a special and dedicated laboratory for cryobiology, clonogenic assays, flow cytometry and fluorescent in situ hybridisation to support ongoing research and development (P Jacobs, L Wood — unpublished data).

In 1995 14 conventional bone marrow transplants were carried out, approximately half being autologous. Five patients (35.7%) died. There were no immediate procedure-related deaths. Clearly there was no technical or any other impediment to providing such tertiary-level medicine successfully in the private sector. However, the number is too small for designation as a transplant centre, while deaths were skewed by sub-optimal selection of cases treated.

In 1996 the number of transplants rose to 27. Twenty-five were carried out using bone marrow, of which 8 were allogeneic. A further 3 were from blood stem cells harvested from matched siblings and, at the time, represented a further innovation in this country. Six patients (22.2%) died, mostly from disease progression. This current level of activity is appropriate for designation as a transplant centre, using international criteria. The falling morbidity reflects improved patient selection and more cohesive functioning of the nursing and paramedical support staff. The switch to the use of peripheral blood has significantly reduced costs and shortened hospital stay, and has had much greater acceptability by family donors.

1997 saw the programme reach capacity, with approximately one graft carried out each week. At the time of reporting, 6 patients who received allografts and 5 who received autographs have died. For the first time cytomegaloviral pneumonitis was noted, leading to introduction of appropriate monitoring as a basis for early therapy with ganciclovir.

Children are now routinely treated and an increasing number of solid tumours are managed on international collaborative protocols. Of particular note has been the successful introduction of matched unrelated transplants, and these are used where appropriate. A new problem has been the low-level occurrence of erythroderma, presumed to be grade 1 cutaneous graft-versus-host disease and generally readily responsive to corticosteroid immunosuppression. It is postulated that such an immunological event might carry with it a desirable graft-versus-leukaemia benefit which may contribute to a continually low relapse rate.

In 1998 51 transplants have been completed, of which approximately half are allogeneic. Engraftment time has been shortened by over a week to 11 days, and there have been no graft failures. At the time of reporting 8 patients have died. Two were disease-free with a functioning graft, 2 died of strokes, and 1 died of pulmonary embolus. The other deaths were due to a variety of causes.

In 1999 the same level of activity and pattern of response has been maintained, with 38 allogeneic and 27 autologous peripheral blood stem cell grafts successfully undertaken. The year was notable for the increased number of children qualifying for admission to protocol studies and rapidly escalating activity in the matched unrelated volunteer donor transplantation. Many of the initial problems with establishing donor searches and the logistics of safe as well as rapid delivery of appropriate products for the procedures have been overcome. An important advance has been the acceptance of the centre, both for transplantation and harvesting, by the American National Donor Programme. This will markedly broaden the range of available options.

The priorities for the first 5 years of the new millennium are clear. Firstly, to provide a service in keeping with international
standards of practice. Secondly, to refine graft quality and investigate the phenomenon of erythrophagia. Thirdly, to continue studies to characterise recovery of immunological competence, and to extend observations from the CAMPATH users group on an apparent benefit of post-transplant administration of cyclosporin (G Hale — unpublished data). We will also consolidate our increasing use of unrelated volunteer transplants through continued association with the South African and international bone marrow donor registries. Finally, the stage is now set to commence development of refinements such as gene therapy for the more selective correction of defects exemplified by Fanconi anaemia, and here the necessary collaboration with an international accredited laboratory for DNA engineering has been established. In these endeavours we will explore ways of developing collaborative programmes with the State hospitals so that community resources are utilised maximally and responsibly for adults and children. Only through such mutual endeavours will the greatest number of individuals be able to benefit from costly, but nevertheless frequently curative interventions.

We conclude by re-emphasising our commitment to accountability and audit, particularly now that managed health care is increasingly becoming a reality. We respectfully submit, therefore, that peer review must continue to govern these activities, so that all potentially eligible patients can continue to have access to a procedure that, while expensive, is both cost-effective and frequently curative if properly used.

P Jacobs
L Wood

Department of Haematology and Bone Marrow Transplant Unit
Constantiaberg Medi-Clinic
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Cape Town

10. Link H, Schmid N, Gratwohl A. Goldman J. For the Amsterdam Sub-Committee of the European Group for Blood and Marrow Transplantation (EBMT). Standards for specialist units undertaking blood and marrow stem cell transplants: recommendations from the EBMT. Bone Marrow Transplant 1990; 5: 725-736.

REDDUCING MOTHER-TO-CHILD TRANSMISSION OF HIV

To the Editor: As health workers in government service, we face a dilemma regarding the care of pregnant women infected with HIV. The best available evidence indicates that various relatively inexpensive short courses of antiretroviral therapy are highly effective in reducing mother-to-child transmission of HIV. The Department of Health has chosen not to make this preventive measure available to women in the public health sector, or to inform women of the available evidence so that they have the option to obtain treatment themselves.

Background
In 1998 a pilot study for which funding was available was planned by the Gauteng Health Department to determine the feasibility and cost of implementing a strategy to reduce mother-to-child transmission of HIV at all levels of health care. We supported this programme, which aimed at equity throughout the health service. As part of the pilot study, a system of antenatal counselling and voluntary HIV testing was set up at Coronation Hospital and a primary care referral clinic. After several months of meetings to finalise the protocol, the pilot study was cancelled. We protested in writing about this decision.

While we accept that some therapies cannot be afforded in the public sector, we considered it unethical to withhold from those in our care a life-saving preventive measure without even assessing whether or not it is affordable for all. We therefore decided to make savings in other areas in order to make short-term antiretroviral therapy available to women attending our hospital.

Our experience has been overwhelming positive. Our staff have implemented the programme with such enthusiasm and dedication that our antenatal HIV clinic was jointly awarded the 1999 Gauteng Province Kanyisa award for service excellence. The sense of helplessness of staff and patients in the face of the HIV epidemic has turned to a sense of purpose and optimism, that at least in one area we are able to make a positive contribution to curbing the epidemic. Several women attending our clinic have been trained as lay HIV counsellors. We see the positive approach to the prevention of mother-to-child transmission of HIV as a catalyst for changing community attitudes from hopelessness and denial to a determination to do something about the HIV epidemic.

The question of safety and effectiveness
We have re-evaluated our position in the light of recent government statements regarding safety. Our position is as follows:

1. All drugs have the potential for adverse effects, which must be weighed against the beneficial effects. In the case of short-term courses of AZT, ACT plus 3TC, or nevirapine to pregnant women infected with HIV and their newborn babies,
considered to have had a recurrence at day 1. Leukemia-free survival was defined as survival in continuous complete remission; relapse or death were considered events, and patients surviving in continuous complete remission were censored at last follow-up.

**Statistical Methods**

Patient-, disease-, and transplant-related variables were compared between the two groups using the $\chi^2$ statistic for categorical variables and the Kruskal-Wallis test for continuous variables. Probabilities of leukemia-free survival and overall survival were calculated using the Kaplan-Meier estimator. Cumulative incidence rates (the chance a patient will have experienced a particular event before time $t$, and where death without an event is the competing risk) were calculated using standard technique, for hematopoietic recovery, acute and chronic GVHD, treatment-related mortality, and relapse. We calculated 95% CIs using the SE of the survivor function by Greenwood formula. Adjusted probabilities for outcomes after transplantation were estimated using the Cox proportional hazards method to adjust for patient-, disease-, and transplant-related variables that were included in the final multivariate models.

Multivariate models were built using a stepwise forward selection with a significance level of 0.05. The primary objective of this study was to compare outcomes after PBSC and BM transplantation; therefore, the variable for graft type was held in the model at each step. Other variables considered were recipient age, performance status, sex of the recipient and donor, age of recipient and donor, year of transplantation, conditioning regimen, type of prophylaxis against GVHD, and use or nonuse of growth factor within the first 7 days of allograft infusion to hasten neutrophil recovery. Whenever categories of variables initially classified into more than two categories showed no statistically significant differences between categories, categories were collapsed to create the fewest possible number of groups (such as age 8 to 16 years v 17 to 20 years). Variables were entered using a time-varying covariate method to determine whether the proportional hazards assumption was met. All variables met the proportional hazards assumption. First-order interactions between graft type and each variable of interest were examined by fitting a proportional hazards model, and examining the interaction between the variable of interest and graft type. All multivariate models were examined for center effects using a random effects or frailty model. Completeness of follow-up was assessed using the $\gamma$ statistic, which gives a measure of the proportion of all potential follow-up information available for this study. All $P$ values are two-sided. All analyses were done using PROC PHREG in SAS version 8.0 (SAS Institute, Cary, NC).

**Patient and Transplant Characteristics**

Patient-, disease-, and transplant characteristics are presented in Table 1. All aspects of the transplant regimen, including graft type, were at the discretion of the transplant center. Median follow-up of survivors was 57 months (range, 4 to 92 months) and 40 months (range, 6 to 82 months) after BM and PBSC transplantation, respectively. Completeness of follow-up was 91% and 94% after BM and PBSC transplantation, respectively. Groups were similar in terms of patient sex, performance score, disease, French-American-British subtypes in AML, and immunophenotype in ALL, cytogenetics, conditioning regimen, GVHD prophylaxis, cytomegalovirus serology, and donor-recipient sex match. Groups differed in distribution of age, disease status at transplantation, use of growth factor, total nucleated cell dose, and year of transplantation (PBSC recipients were older and more likely to have advanced leukemia [be in second CR or not in CR at transplant], more likely to receive growth factors and higher cell doses, and have undergone transplantation more recently).

**Donor Characteristics**

PBSC donors tended to be older than BM donors (Table 1). Donor age was highly correlated with recipient age in both groups ($P < .0001$). Data on method of PBSC collection were available for 108 donors. Of these, 19% required placement of a central venous catheter. No collection-related complications were reported in 137 assessable PBSC donors. BM grafts were collected using standard techniques. There were four reported complications among BM donors: hypotension, nausea, vomiting, allergic reaction, and pyelonephritis, as reported verbatim on IBMTR report forms.

**Hematologic Recovery**

PBSC recipients had significantly faster neutrophil and platelet recovery. Median time to neutrophil recovery was 13 days (range, 7 to 34 days) and 18 days (range, 9 to 47 days) after PBSC and BM transplantation, respectively ($P < .0001$, Kruskal-Wallis test). The cumulative incidence of neutrophil recovery at day 100 was 97% (95% CI, 82% to 100%) and 96% (95% CI, 90% to 99%) after PBSC and BM transplantation, respectively. Median time to platelet recovery was 18 days (range, 8 to 92 days) and 26 days (range, 11 to 117 days) after PBSC and BM transplantation, respectively ($P < .0001$, Kruskal-Wallis test). The cumulative incidence of platelet recovery at 1 year was 87% (range, 82% to 92%) and 88% (range, 83% to 93%) after PBSC and BM transplantation, respectively.

**Acute and Chronic GVHD**

Cumulative incidences of grades 2 to 4 acute GVHD at day 100 were 27% (95% CI, 20% to 35%) and 28% (95% CI, 25% to 32%) after PBSC and BM transplantation, respectively. Cumulative incidences of grades 3 to 4 acute GVHD at day 100 were 13% (95% CI, 8% to 20%) and 11% (95% CI, 8% to 13%) after PBSC and BM transplantation, respectively. In multivariate analysis, the relative risk (RR) of grades 2 to 4 acute GVHD were similar after PBSC and BM transplantation (RR, 1.01; 95% CI, 0.72 to 1.43; $P = .9$). Results were similar for grades 3 to 4 acute GVHD. In both groups, acute GVHD was significantly higher after an irradiation-containing conditioning regimen (RR, 1.60; 95% CI, 1.21 to 2.13; $P = .001$).
### Table 1: Patient, Disease, and Transplant Characteristics

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<th>Variable</th>
<th>Total No.</th>
<th>%</th>
<th>BM Transplantation No.</th>
<th>%</th>
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(continued on following page)
### Table 1. Patient, Disease, and Transplant Characteristics (continued)

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Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; CR, complete clinical remission; PIF, persistent induction failure; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulfan; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage-colony stimulating factor, CSA, cyclosporine; MTX, methotrexate, NS, nonsignificant; BM, bone marrow graft; PB, peripheral blood graft; GVHD, graft-versus-host disease; Cy, cyclophosphamide.

*FAB subtypes, immunophenotypes, cytogenetics and disease status: ALL, B-lineage (88% of PBSC and 63% of BM recipients); T-lineage (15% and 23%); undifferentiated (17% and 14%); ALL cytogenetic abnormality, normal karyotype (34% of PBSC and 34% of BM recipients); 19q21, 11q23, 11q24, hyperdiploid (10% and 11%); 11q23, 11q24, 11q22, 10, -12, +21, +19, +14q, hyperdiploid (23% and 29%); AML: M1 (33% of PBSC and 16% of BM recipients); M2 (33% and 34%); M3 (5% and 9%); M4 (24% and 22%); M5 (9% and 11%); M6 (4% and 1%); M7 (2% and 2%); undifferentiated (7% and 5%); AML cytogenetic abnormality, 19q21, 11q23, 11q24, 19q13 of PBSC and 16% of BM recipients; 16q, 18q, 16q, 11q, 8, +21, normal karyotype (30% and 31%); 11q23, 11q22, 11q24, monosomy 7, 7q, 7q, -20/20q, -16q, and 4q; unknown (33% and 29%).

1) Disease status: CR1 at transplantation, ALL: n = 32 PBSC and n = 119 BM; AML: n = 26 PBSC and n = 211 BM; CR2 at transplantation, ALL: n = 42 PBSC and n = 197 BM; AML: n = 10 PBSC and n = 39 BM; Relapse/PIF at transplantation, ALL: n = 14 PBSC and n = 40 BM; AML: n = 17 PBSC and n = 26 BM.

2) Other conditioning regimen BM, cyclophosphamide; other (n = 5), busulfan; other (n = 1), thiotepa; other (n = 4), TBI.

3) Other GVHD prophylaxis: FK506, methotrexate, - steroids.
4) Use of growth factor within the first 7 days after transplantation: BM, G-CSF (n = 133); GM-CSF (n = 15) and G-CSF: GM-CSF (n = 5); PB, G-CSF (n = 59); GM-CSF (n = 3) and G-CSF + GM-CSF (n = 1); missing (n = 6).

Cumulative incidences of chronic GVHD at 3 years were 33% (95% CI, 24% to 42%) and 19% (95% CI, 16% to 22%) after PBSC and BM transplantation, respectively (P = .001; Fig 1). In a multivariate analysis, chronic GVHD was significantly more likely after PBSC transplantation (RR, 1.84; 95% CI, 1.28 to 2.64; P = .001). Other factors significantly associated with chronic GVHD in both groups were age older than 16 years (RR, 1.66; 95% CI, 1.21 to 2.29; P = .002) and donor-recipient sex match other than female donor and male recipient (RR, 0.61; 95% CI, 0.44 to 0.84; P = .003). Among patients with chronic GVHD, grade and organ involvement were similar after PBSC and BM transplantation (Table 2).

### Treatment-Related Mortality

Cumulative incidences of treatment-related mortality at 3 years were 26% (95% CI, 19% to 34%) and 14% (95% CI, 11% to 17%) after PBSC and BM transplantation, respectively (P = .001; Fig 2). Treatment-related mortality was higher after PBSC transplantation (Table 3). The use of growth factor was significantly associated with higher treatment-related mortality after both PBSC and BM transplantation.

### Relapse

Cumulative incidences of relapse at 3 years were 38% (95% CI, 29% to 47%) and 33% (95% CI, 29% to 37%) after PBSC and BM transplantation, respectively (P = .70). Risks of relapse were similar in both groups (Table 3). Advanced disease at transplantation and use of growth factor were
significantly associated with an increased risk of relapse after both PBSC and BM transplantation. We observed a trend toward a protective effect of chronic GVHD in preventing relapse after both PBSC and BM transplantation (RR, 0.69; 95% CI, 0.47 to 1.02; P = .06).

Leukemia-Free Survival

Treatment failure (inverse of leukemia-free survival) was higher after PBSC than after BM transplantation (Table 3). Advanced disease at transplantation and use of growth factor were significantly associated with higher treatment failure after both PBSC and BM transplantation. Three-year probabilities of leukemia-free survival, adjusted for other significant factors (disease status and use or nonuse of growth factor), were 42% (95% CI, 33% to 50%) and 51% (95% CI, 48% to 55%) after PBSC and BM transplantation, respectively (P = .03; Fig 3).

Overall Survival

Mortality was higher after PBSC than BM transplantation (Table 3). Advanced disease and use of growth factor were significantly associated with higher mortality after both PBSC and BM transplantation. Three-year probabilities of overall survival, adjusted for other significant factors, were 48% (95% CI, 40% to 56%) and 58% (95% CI, 54% to 62%) after PBSC and BM transplantation, respectively (P = .01; Fig 4).

Eighty-two (57%) of 143 recipients of PBSC transplants and 277 (44%) of 630 recipients of BM transplants died (Table 4). Recurrent disease was the most frequently
reported primary cause of death in both groups. The only significant difference in causes of mortality between PBSC and BM recipients was in the proportion of deaths related to GVHD occurring more than 100 days post-transplantation—20% in the PBSC group and 5% in the BM group ($P = .0007$, $\chi^2$ test).

Several studies, including randomized clinical trials comparing PBSC versus BM transplantation in adults, report lower early mortality, higher chronic GVHD risk, and a survival benefit in some populations. Though there are no such studies reported in children, PBSC allografts are frequently used instead of BM for pediatric transplantation. The primary objective of this study was to compare outcomes after transplantation using PBSC and BM allografts in children and adolescents with acute leukemia. There were differences in PBSC and BM recipients that might be expected to confound outcomes after transplantation, such as age, type of leukemia, disease status at transplantation, and use of growth factor. We used multivariate Cox regression models to adjust for the independent effects of each potential risk factor. After this adjustment, we found higher chronic GVHD, treatment-related mortality, treatment failure, and overall mortality after PBSC transplantation.

PBSC offer more rapid hematopoietic recovery in children, similar to reports in adults. We also observed similar risks of acute GVHD after PBSC and BM transplants, which was again, comparable to most reports in adults. As observed in some studies of adults, chronic GVHD was significantly higher after PBSC transplantation, probably due to higher numbers of T cells in mobilized peripheral blood. However, among patients who developed chronic GVHD, grade and organ involvement were comparable in the two groups, consistent with reports in adults.

Importantly, we observed an adverse effect of PBSC grafts on treatment-related mortality, treatment failure, and overall mortality after adjusting for other significant risk factors that may influence outcome. This has not been reported previously. Published reports in adults with acute leukemia suggest equivalent survival after PBSC and BM transplants. Higher mortality in children receiving PBSC grafts is likely due to higher chronic GVHD. Chronic GVHD after BM transplantation is lower in children than in adults. Thus in younger PBSC recipients, a higher risk of mortality from chronic GVHD may offset any measurable benefits of more rapid hematopoietic recovery in the early transplantation period.

Higher mortality and treatment failure after PBSC transplantations were independent of age. To address whether the higher proportion of children (aged 17 to 20 years) in the PBSC group affected outcomes, we first examined the effect of age within each graft type and observed no differences in risks of treatment-related mortality, treatment failure, and overall mortality between children aged 8 to 16 years and 17 to 20 years. In multivariate analysis, risks of each of these outcomes after PBSC and BM transplantation were similar in recipients aged 8 to 16 years and 17 to 20 years (i.e., the PBSC/age 8 to 16 years vs BM 8 to 16 years, and the PBSC age 17 to 20 years vs BM/age 17 to 20 years). Formal tests for interaction revealed no significant interactions between graft type and age at transplantation.

Risks of relapse were similar after PBSC and BM transplantation. Thus far, previous studies in adults including randomized clinical trials, also report similar risks of relapse after PBSC and BM transplantations. The observed trend toward a protective effect of chronic GVHD in preventing relapse did not differ by graft type. Our study is limited to patients with acute leukemia, whereas reports that have suggested a stronger graft-versus-leukemia effect after PBSC transplantations have included patients with acute and chronic leukemia.

Disease status at transplantation adversely affected leukemia recurrence, treatment failure, and overall mortality, regardless of the type of graft. Though a higher proportion of patients with ALL underwent transplantation in second
Table 4. Primary Causes of Early and Late Death After Bone Marrow and Peripheral-Blood Stem-Cell Transplantations.

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<tr>
<td>Infection</td>
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<td>26</td>
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<tr>
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<td>13</td>
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Abbreviation: GvHD, graft-versus-host disease.

CR, this was independent of graft type. In contrast, most patients with AML were transplanted in first CR but were more likely to receive PBSC grafts if they were not in CR at transplantation. We did not observe an association between transplant outcomes and disease type.

Use of growth factor for neutrophil recovery had an adverse effect on relapse and mortality after PBSC and BM transplantations. Studies in animals and human volunteers have shown decreased production of inflammatory cytokines, increased production of interleukin-10, increased mobilization of T-helper-2 (Th2)-inducing dendritic cells, and Th2 immune deviation with G-CSF. In recipients of mismatched T-cell-depleted transplantations, the elimination of G-CSF for hematopoietic recovery has been shown to accelerate immune recovery. It is possible that the use of growth factor may have resulted in slower immune recovery and subsequent higher mortality. We were unable to explain the adverse effect of growth factor on leukemia recurrence. Thus far, larger studies specifically examining the effect of growth factor use in the early post-transplantation period, report mixed results, and although growth factors activate AML cells, most reports support the contention that leukemia growth is not promoted in a clinically meaningful way. A detailed analysis of the effects of growth factor on leukemia recurrence or mortality is beyond the scope of this study.

We did not perform analysis of total nucleated cell dose or CD34 cell dose, as these were surrogates for graft type. PBSC recipients received higher cell doses: 65% received total nucleated cell doses greater than 5 x 10⁸/kg compared with 10% of BM recipients. Nevertheless, most BM recipients received an adequate cell dose and achieved hematopoietic recovery, and this may have minimized any potential benefit of PBSC grafts. We could not analyze the independent effect of donor age as this was highly correlated with recipient age in both groups.

We found no evidence of confounding of main effects (transplant outcome by graft type) by center effects using a random effects model, a test specially designed for situations like the current analysis in which each center contributes relatively few cases. To address this further, we adjusted for the number of transplantations performed per center (1 to 5 vs. 10 to 15 vs. 10) and geographic location (United States vs. Europe vs. other regions) in final multivariate models and found no evidence of confounding of main effects.

Treatment-related mortality, treatment failure, and overall mortality were higher after PBSC transplantation after adjustment for other relevant risk factors that may influence these end points. A retrospective analysis has limitations such as selection bias for graft type and inability to adjust for unknown or unmeasured factors. These data represent the early experience of PBSC transplantation; nevertheless, these data should serve a cautionary note before widespread change in the clinical practice of using BM for allogeneic transplantation in children. Alternative graft sources must be evaluated in a controlled fashion before adoption in a population that already has a relatively good outcome after BM transplantation. We think that there is an urgent need for a properly designed clinical trial to define the role of PBSC in allogeneic transplantation in children.

Appendix

In addition to the authors, other members of the International Bone Marrow Transplant Registry Working Committee on Histocompatibility and Alternate Stem Cell Sources who participated in the study are as follows: M.M. Abecasis, Instituto Portugues de Oncologia, Lisbon, Portugal; B.J. Bowell, Cleveland Clinic Foundation, Cleveland, OH; K.W. Chan, M.D. Anderson Cancer Center, Houston, TX; J. Davis, British Columbia Children's Hospital, Vancouver, BC, Canada; M.A. Diaz, Hospital Nino Jesus, Madrid, Spain; J. Doyle, Hospital of Sick Children, Toronto, ON, Canada; M. Gorner, University of Heidelberg, Heidelberg, Germany; G.A. Hale, St Jude Children's Research Hospital, Memphis, TN; R.E. Harris, Cincinnati Children's Hospital Medical Center, Cincinnati, OH; M. Kretzel, Children's Memorial Hospital, Northwestern
University Feinberg School of Medicine, Chicago, IL; C.F. LeMaistre, Texas Transplant Institute, San Antonio, TX; D.I. Marks, University of Bristol, Bristol, UK; J.A. Martinez, La Fe University Hospital, Valencia, Spain; M. Mazziati, Oregon Health Sciences University, Portland, OR; S.R. McCann, St James’s Hospital, Dublin, Ireland; P.S. Negrin, Stanford University Medical Center, Stanford, CA; and J.J. Ortega, Hospital Infantil Vall d’Hebron, Barcelona, Spain.

Authors’ Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.
Primed marrow for autologous and allogeneic transplantation: A review comparing primed marrow to mobilized blood and steady-state marrow

Gerald J. Elfenbein and Robert Sackstein

Mobilized peripheral blood collections, obtained following either chemotherapy (with or without granulocyte colony-stimulating factor (G-CSF)) or G-CSF administration alone, are rapidly replacing traditional bone marrow harvests as the source of cells for hematopoietic stem cell transplantation. According to the Autologous Blood and Marrow Transplant and the International Bone Marrow Transplant Registries, for the years 1998 through 2000, blood stem cell (BSC) transplants accounted for about 80% of autologous transplants in the pediatric age group and more than 90% of the autologous transplants among adults. In allogeneic transplantation, where the donor is a healthy family member or normal volunteer, G-CSF-mobilized BSC transplants are being used more and more frequently, accounting for about 20% of allogeneic transplants in the pediatric age range and more than 40% of allogeneic transplants among adults during the same time period. It is not, therefore, too great a stretch to imagine that BSC transplants will soon be, if not already, in the majority for allogeneic transplantation among adults. The principal reason why this is happening is the prevailing view that BSC engraft more rapidly than marrow stem cells (MSC). However, this view is based on comparisons between primed circulating blood cells (BSC) and unprimed resident marrow cells in the steady state (SS-MSC). If the reason why BSC engraft faster than SS-MSC were a consequence of G-CSF used for mobilization, then would priming of MSC by G-CSF (Prim-MSC) accelerate engraftment of marrow as well? We reviewed the literature of the last 10 years to see if there were enough data to answer this question.

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During embryogenesis, hematopoietic stem cells migrate from the yolk sac to the liver and, ultimately, to the medullary cavities of cancellous bones. This migration occurs via the vasculature and, in adult animals, there are persistent but low levels of migrating hematopoietic stem cells in the blood in the steady state. In a canine lethal irradiation model, cross-circulation of blood from an allogeneic dog produced engraftment of donor hematopoiesis as documented by chimeraism [1]. In another canine myeloablative model, allogeneic leukocyte transusions successfully repopulated marrow spaces and blood elements [2]. These two studies, as well as many others, formally demonstrated the repopulating capacity of circulating hematopoietic stem cells in the bloodstream of large animals.

In man, Goldman [3] reported the first use of blood (albeit Philadelphia chromosome (Ph)-positive) cells for autologous transplantation after high-dose cytotoxic therapy in patients with chronic myelogenous leukemia (CML). Köbbing et al. [4] reported the first case of Ph-negative, autologous blood cell transplantation, also in a patient with CML. In a subsequent case report of a patient with Burkitt’s lymphoma, Köbbing et al. [5] introduced the hypothesis that autologous BSC may produce more rapid engraftment than autologous MSC. In both reports by Köbbing, BSC were collected from patients during the overshoot phase of granulocyte-macrophage colony-forming units in the blood during leukocyte recovery after cytotoxic chemotherapy. This was the first example of chemotherapy-induced “mobilization,” one of the two major methods we employ today to enrich autologous stem cells in the blood [6].

The modern era of autologous BSC transplantation, however, began with the report by Kessinger et al. [7], which
documented, with multiple leukaphereses, the presence of sufficient numbers of autologous hematopoietic stem cells in steady-state blood (long after recovery from chemotherapy) to ensure engraftment after myeloablative therapy. Thus, there is little question that adequate hematopoietic progenitors may be obtained from the bloodstream for hematologic recovery after myeloablative treatment.

Growth factor–induced mobilization is the second of the two major methods for enriching stem cells in the bloodstream [8]. G-CSF is widely used to mobilize BSC for transplantation. Numerous reports have hypothesized that G-CSF–mobilized BSC (BSC) engraft more rapidly than steady-state MSC (SS-MSC), much as Körbling had suggested for chemotherapy BSC. Because of the wide acceptance of this hypothesis, G-CSF BSC are being collected more and more often from healthy donors (including unrelated volunteer adult donors) for autologous transplantation after myeloablative therapy [9].

Recent reports concerning the induction of proteolysis in the marrow microenvironment by G-CSF have shed light upon what may be the molecular mechanism of growth factor mobilization of progenitor cells [10–13]. G-CSF induces proteolysis of at least CD106 (vascular cell adhesion molecule-1 or VCAM-1), which results, subsequently, in the release of progenitors, capable of repopulating hematopoiesis into the circulation. What remains unclear at present is why BSC would produce more rapid engraftment than SS-MSC. This review may shed light upon this open question.

Literature search
The search for articles published over the last 10 years in peer-reviewed, English-language journals and authoritative, English-language monographs comparing BSC and MSC was initiated with PubMed on-line. The search was predicated upon the key words: autologous or allogeneic or HLA-identical; transplantation; stem cell; and blood and/or marrow. Several searches at different times putting the key words in different orders were performed. To be included in our analyses, published articles must have reported comparisons of at least two of the three different types of stem cell products—BSC, SS-MSC, and Prim-MSC. Reference lists from all publications so obtained were also reviewed for additional publications germane to the topic that were not detected via PubMed on-line. Published meeting abstracts were excluded from the analyses performed because of the risk of incomplete data. The search concluded in January 2003.

Although meta-analyses are the most sensitive way to detect small differences between study groups, due to the number and heterogeneity of reports examined, it was not feasible to obtain primary data to execute meta-analyses. As an alternative statistical method, which takes into account differences in sample size for different studies, we calculated and report here weighted median stem cell numbers and weighted median days to engraftment of granulocytes (the first of three days when the absolute granulocyte count (AGC) exceeded 0.5 × 10^9/L) and platelets (the first of three days when the platelet (PLT) count exceeded 20 × 10^9/L unsupported by platelet transfusions). Medians were weighted by sample size. In addition, we calculated and report here weighted median T cell numbers and weighted probabilities of five major clinical outcomes: 1) acute graft-versus-host disease (GVHD; Grade II or higher), 2) chronic GVHD (extensive and limited), 3) relapse rate (100% minus percentage of patients, at the median follow-up time, remaining relapse free from Kaplan-Meier time to relapse plots), 4) overall survival (percentage of patients, at the median follow-up time, remaining alive from Kaplan-Meier time to death plots), and 5) disease-free survival (percentage of patients, at the median follow-up time, remaining both free of relapse and alive from Kaplan-Meier time to event plots).

In this report, we term inter-study comparisons “global” analyses. Also, we term intra-study comparisons “delta” analyses. Intra-study delta analyses minimize the contribution of patient heterogeneity that is introduced by evaluating multiple studies at the same time, to each specific endpoint measurement. The assumption made by delta analyses is that patient heterogeneity for both types of stem cell products in a given study will be very similar. Statistical analyses were performed using Statistica software (Tulsa, OK, USA). Medians for granulocyte and platelet engraftment times were not expected to be normally distributed. Assuming nonparametric distributions of medians, comparisons among stem cell product groups were performed using the Kruskal-Wallis ANOVA and median test. Differences between the medians for engraftment times for two different stem cell products were expected to be normally distributed. The null hypothesis was that the average of deltas was zero. The Z test was used to test the null hypothesis. Delta analyses were performed for the subgroup of studies that were randomized controlled trials first, then, if necessary, for all studies comparing two stem cell products “head-to-head.” To determine risk factors for individual outcomes, we employed an ANCOVA method. All p-values presented are from two-tailed tests.

Results

Literature review
The objective of the literature search was to assemble as many articles as possible from the modern literature (past 10 years) to evaluate seven clinical outcomes, i.e., engraftment times for granulocytes and platelets, acute and chronic GVHD, relapse rate, overall survival, and disease-free survival. The ultimate goal of the literature review was to determine if there were differences in any of these seven
clinical outcomes for both autologous and allogeneic transplants based upon anatomic source of stem cells and/or use of G-CSF before collection. As will be seen, the results provide a new perspective to help us formulate a more accurate hypothesis about engraftment relating to the anatomic source of stem cells and the use of G-CSF precollection of BSC (mobilization) and MSC (priming).

Thirty-seven articles were collected, 13 reporting autologous transplants [14-26] and 24 reporting allogeneic transplants [27-50]. It was important to identify articles that reported results not only for comparisons of BSC with SS-MSC but also for comparisons of Prim-MSC with either BSC or SS-MSC, as well.

Autologous transplantation

In the autologous stem cell transplant literature, there were 18 eligible comparisons in the 13 reports. There was considerable heterogeneity among the 18 comparisons. There were 9 prospective comparisons (6 with randomization, 2 with assigned controls, and 1 with concurrent controls) and 9 retrospective comparisons (4 with historical controls, 4 with matched controls, and 1 with sequential patients). Eleven comparisons were from single institutions; 12 comparisons involved lymphoma patients only; 7 comparisons involved a single transplant regimen; and 16 comparisons involved adults only. Eleven comparisons used growth factors during the first 7 days after stem cell infusion (9 with G-CSF and 2 with GM-CSF); growth factors were not administered in 5, and there was no mention of postgrafting growth factors in 2 comparisons.

A grand total of 407 patients with BSC were described. Mobilization of BSC was performed in 16 comparisons involving growth factors in 10 (9 with G-CSF and 1 with GM-CSF), employing chemotherapy and growth factors in 2, and not described in 4 comparisons. When G-CSF was used to mobilize BSC, the median (range) dose was 10 (5-16) μg/kg/day for 4 (4-5) days followed by 2 (1-4) leukaphereses. Mobilization of BSC yielded a weighted median (range) of 2.6 (1.0-6.4) x 10⁸ CD34+ cells/kg.

There were 12 comparisons involving SS-MSC describing 347 patients. The weighted median (range) harvest of CD34+ cells was 2.1 (1.2-2.3) x 10⁹/kg. There were 9 comparisons involving growth factor Prim-MSC (8 with G-CSF and 1 with GM-CSF) reporting 159 patients. When G-CSF was used to prime MSC, the median (range) dose was 10 (1-40) μg/kg/day for 5 (2-5) days followed by a single bone marrow harvest. Priming of MSC yielded a weighted median (range) of 1.5 (0.6-2.3) x 10⁹ CD34+ cells/kg.

Figure 1 is a graphic presentation of four outcomes of autologous transplants: recovery of granulocytes and platelets, overall survival, and disease-free survival. For the first round of analyses no study was excluded. This round was "global" in nature. As can be seen, there was little difference in the numbers of CD34+ cells infused/kg among the three groups—BSC, SS-MSC, and Prim-MSC. The top-to-bottom spread was 2.6 to 1.5 x 10⁹ CD34+ cells/kg, representing a spread of less than a factor of 2. Both AGC and PLT recoveries were, however, delayed for SS-MSC and Prim-MSC as compared to BSC. Prim-MSC recoveries were more rapid than SS-MSC, demonstrating a salutary effect of growth factor priming on MSC despite the fact that Prim-MSC contained about 25% fewer CD34+ cells (per kilogram recipient body weight) than did SS-MSC. Interesting is the observation that Prim-MSC engraft nearly as rapidly as BSC despite having 40% fewer CD34+ cells than BSC. Overall survival at a median follow-up of 3.6 years was essentially identical for BSC and SS-MSC. Disease-free survival at a median follow-up of 2.8 years was similar for all three classes of stem cells with a potential edge for MSC. Altogether this global analysis would speak to faster engraftment for BSC, which would translate into briefer aplasias, reduced infections, and fewer transfusions resulting in lower costs, but without an ultimate long-term survival benefit [51-52].

However, because of the extraordinary degree of heterogeneity among the comparisons, as described above, we felt that the global analysis was, by itself, insufficient to draw conclusions. The second round of analyses ("delta" analyses) evaluated only prospective randomized controlled trials (RCT). In this round, analysis of engraftment involved calculating the delta (signed days of difference in the medians) in engraftment times between the two arms of the RCT. There were 4 RCT [14,16,18,26] that compared BSC and SS-MSC and 2 RCT [19,24] that compared BSC to Prim-MSC. There were no RCT comparing SS-MSC or Prim-MSC. When delta is positive, it signifies faster recovery for BSC relative to its comparator (SS-MSC or Prim-MSC); when delta is negative, the reverse is true. Although a delta
of +1 day for AGC recovery may be mathematically significant due to the tightness of distribution of engraftment times [53]. Such a delta is not clinically meaningful because it signifies, usually, only one more day of antibiotics. Similarly, a delta of +2 days for PLT recovery may be mathematically significant but not clinically meaningful because it represents, ordinarily, only one more platelet transfusion. Figure 2A shows that BSC produced more rapid granulocyte engraftment than SS-MSC in 3 of 4 RCT. Further, in 2 of 2 RCT, BSC and Prim-MSC produced equivalent engraftment times. Figure 2B shows that BSC produced more rapid platelet engraftment than SS-MSC in 3 of 4 RCT. Further, in 2 of 2 RCT, BSC and Prim-MSC produced equivalent engraftment times. In contrast to the global analysis above, this RCT analysis points to equivalence of engraftment potential for both autologous MSC and BSC collected after G-CSF treatment. Furthermore, with respect to overall and disease-free survival, 5 RCT were evaluable. Four of 5 RCT showed equivalence of overall survival while 1 of 5 showed an advantage for BSC [14]. All 5 RCT showed equivalence of disease-free survival for MSC and BSC.

Allogeneic transplantation

In the allogeneic stem cell transplant literature, 24 manuscripts report eligible comparisons. There was considerable heterogeneity among the 24 comparisons. There were 10 randomized prospective studies, 5 studies with historical controls, 4 retrospective registry studies, 2 studies with case controls, 2 sequential studies, and 1 allocated study. Fifteen comparisons were from single institutions; only 2 comparisons involved only one institution (but not the same one); only 7 comparisons involved a single transplant regimen; but 22 comparisons involved adults only. Acute GVHD prophylaxis involved cyclosporine in 22 studies (with 2 making no mention). In addition, methylprednisolone was given to all patients in 15, some patients in 6, and no patients in 2 studies, but was not mentioned in 1 study. G-CSF use during the first 7 days after stem cell infusion was routine in 7, sometimes in 4, not routine in 4, not allowed in 4, but not mentioned in 5 studies.

A grand total of 1949 patients with BSC were described. Mobilization of BSC was performed in 22 comparisons; methodology involved G-CSF in 19 but was not reported in 3 comparisons. To mobilize BSC with G-CSF, the median (range) dose was 10 (5-16) µg/kg/day for 4 (3-5) days followed by 2 (1-4) leukaphereses. Mobilization specifics (dose, schedule, duration, and number of collections) were not completely described in 4 comparisons. Mobilization of BSC with G-CSF yielded a weighted median (range) of 6.1 (3.1-12.3) x 10^6 CD34+ cells/kg.

There were 21 comparisons involving SS-MSC describing 3782 patients. The weighted median (range) harvest of CD34+ cells was 3.2 (1.5-7.5) x 10^6/kg for SS-MSC. There were only 5 comparisons involving G-CSF Prim-MSC reporting 107 patients. To prime MSC with G-CSF, the median (range) dose was 10 (4-10) µg/kg/day for 4 (2-7) days followed by a single bone marrow harvest. Priming of MSC yielded a weighted median (range) of 4.1 (1.6-9.4) x 10^6 CD34+ cells/kg. The regimens involving G-CSF treatment prior to collecting BSC and Prim-MSC were virtually identical.

Figure 3 is a graphic presentation of 7 outcomes of allogeneic transplants: recovery of granulocytes and platelets, acute and chronic GVHD, relapse rate, overall survival, and disease-free survival. The medians for recovery of granulocytes and platelets for each stem cell product for all studies

Figure 2. For allogeneic transplants, delta analysis of engraftment times from randomized controlled trials. Panel A shows the number of days for granulocyte recovery (to >0.5 x 10^9/L) that G-CSF-mobilized blood stem cells (BSC) engraft faster than steady state marrow stem cells (SS-MSC) and G-CSF-primed MSC (Prim-MSC). Biologically significant differences are indicated by days more than the arrow marks (in this case, more than one day). Panel B shows the number of days for platelet recovery (to >20 x 10^9/L) that BSC engraft faster than SS-MSC and Prim-MSC. Biologically significant differences are indicated by days more than the arrow marks (in this case, more than two days).

Figure 3. For allogeneic transplants, CD34+ and CD8+ cell count of grafts and clinical outcomes. Results for 1949 patients who received G-CSF-mobilized blood stem cells (BSC). 3782 patients who received steady state marrow stem cells (SS-MSC), and 107 patients who received G-CSF-primed MSC (Prim-MSC) from 24 different studies [27-50] are shown. Two-tailed values of p are given.
are tabulated in Tables 1 and 2. For the "global" round of analyses no study was excluded. As can be seen, there was little difference in the numbers of CD34+ cells infused/kg among the three groups—BSC, SS-MSC, and Prim-MSC. The top-to-bottom spread was 0.2 to 3.2 × 10^6 CD34+ cells/kg, representing a spread of slightly less than a factor of 2. Both AGC and PLT recoveries were delayed for SS-MSC compared to BSC. As seen in autologous transplants, Prim-MSC recoveries after allogeneic transplants were much more rapid than SS-MSC, also demonstrating a beneficial effect of G-CSF priming on MSC. Interestingly, Prim-MSC contained only about 30% more CD34+ cells than SS-MSC. Most remarkably, AGC and PLT recoveries were identical for Prim-MSC and BSC even though Prim-MSC contained about 30% fewer CD34+ cells than BSC.

One significant difference among stem cell products is that BSC contain nearly sevenfold more CD34+ cells/kg than SS-MSC or Prim-MSC. No significant differences for acute GVHD were reported among SS-MSC, BSC, and Prim-MSC. However, SS-MSC produced less chronic GVHD than BSC and Prim-MSC produced even less chronic GVHD than SS-MSC. Despite the differences in the incidence of chronic GVHD, the probability of relapse at a median follow-up of 2.4 years was certainly not higher for SS-MSC or Prim-MSC than for BSC. Overall survival at a median follow-up of 1.9 years was essentially identical for BSC, SS-MSC, and Prim-MSC. Finally, disease-free survival at a median follow-up of 1.9 years was similar for BSC and SS-MSC. (There were insufficient disease-free survival data to report for Prim-MSC.)

Altogether the global analysis would speak to equivalent engraftment potential for both G-CSF BSC and G-CSF Prim-MSC, both of which are superior to SS-MSC. Even though the numbers of patients receiving Prim-MSC are limited (n = 107), it is safe to say that there is no more acute GVHD with Prim-MSC and perhaps less chronic GVHD than with

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**Table 1. Delta analysis of allogeneic trials for granulocyte recovery**

<table>
<thead>
<tr>
<th>Comparison/Author (ref no.)</th>
<th>Design of Controls</th>
<th>No. of Patients in 1st vs 2nd Cohort at Right</th>
<th>Median Day to AGC &gt; 0.5 × 10^9/L</th>
<th>Delta of Medians for AGC</th>
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*Difference in days to AGC > 0.5 × 10^9/L for SS-MSC minus BSC, p < 0.001, BSC faster than SS-MSC.

**Table 2. Delta analysis of allogeneic trials for thrombocyte recovery**

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<th>Comparison/Author (ref no.)</th>
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*Difference in days to AGC > 0.5 × 10^9/L for SS-MSC minus BSC, p < 0.001, BSC faster than SS-MSC.

**Abbreviations:** AGC = absolute granulocyte count; BSC = G-CSF-mobilized blood stem cells; SS-MSC = steady-state marrow stem cells; Prim-MSC = G-CSF-primed marrow stem cells.
Table 2. Delta analysis of allogeneic trials for platelet recovery

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<td>registry</td>
<td>17</td>
<td>28</td>
<td>-</td>
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<tr>
<td></td>
<td>Ringsten [29]</td>
<td>registry</td>
<td>16.7</td>
<td>28</td>
<td>-</td>
<td>12</td>
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<tr>
<td></td>
<td>Nagataoki [48]</td>
<td>historical</td>
<td>12</td>
<td>26</td>
<td>-</td>
<td>14</td>
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<td></td>
<td>overall</td>
<td>averages</td>
<td>14.0</td>
<td>22.1</td>
<td>-</td>
<td>8.15*</td>
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<tr>
<td>BSC vs SS-MSC</td>
<td>Isoda [30]</td>
<td>historical</td>
<td>-</td>
<td>26</td>
<td>20</td>
<td>6</td>
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<tr>
<td></td>
<td>Ji [40]</td>
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<td>-</td>
<td>24</td>
<td>17.5</td>
<td>6.5</td>
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<tr>
<td></td>
<td>overall</td>
<td>averages</td>
<td>-</td>
<td>25.0</td>
<td>18.8</td>
<td>6.25*</td>
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<tr>
<td>BSC vs Prim-MSC</td>
<td>Szer [41]</td>
<td>randomized</td>
<td>19</td>
<td>-</td>
<td>17</td>
<td>-2</td>
</tr>
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<td></td>
<td>Morton [32]</td>
<td>randomized</td>
<td>12</td>
<td>-</td>
<td>14</td>
<td>2</td>
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<tr>
<td></td>
<td>Serody [71]</td>
<td>sequential</td>
<td>13</td>
<td>-</td>
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<tr>
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<td>averages</td>
<td>14.7</td>
<td>-</td>
<td>15.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*For number of patients in each arm, see Table 1.
1 Difference in Days to PLT > 20 x 10^9/L for SS-MSC minus BSC, p < 0.001, BSC faster than SS-MSC.
2 Difference in Days to PLT > 20 x 10^9/L for SS-MSC minus Prim-MSC, Prim-MSC faster than SS-MSC. Sample size too low to be statistically significant.
3 Difference in Days to PLT > 20 x 10^9/L for Prim-MSC minus BSC, p = 0.60, Prim-MSC equivalent to BSC.

Abbreviations: PLT = platelet count; BSC = G-CSF-mobilized blood stem cells; SS-MSC = steady-state marrow stem cells; Prim-MSC = G-CSF-primed marrow stem cells.

BSC. Further, as relapse, survival, and disease-free survival are likely to reflect disease state (remission or relapse number) and transplant regimen as well as the salutary effect of GVHD, it is safe to say that Prim-MSC are likely to produce outcomes as good as BSC. From the global analysis, the only clinically meaningful difference detected between MSC and BSC collected after G-CSF treatment is the reduction in the incidence of chronic GVHD [54].

Again, because of the extraordinary degree of heterogeneity among the comparisons, as described above, a round of delta analyses was performed. In this second round, only prospective RCTs were evaluated. Again, this RCT analysis involved the delta in engraftment times (as described above) between the two arms of the studies. There were 9 RCTs [27,28,38–42,44,45] that compared BSC and SS-MSC but only 1 RCT [32] that compared BSC to Prim-MSC. There were no RCTs comparing SS-MSC to Prim-MSC. By the same criteria (delta > 1 for granulocytes and delta > 2 for platelets) as described above, BSC produced more rapid granulocyte engraftment than SS-MSC in 8 of 9 RCTs. In the sole RCT of its kind, BSC and Prim-MSC produced equivalent granulocyte engraftment times. BSC produced more rapid platelet engraftment than SS-MSC in 9 of 9 RCTs. But in the single RCT, BSC and Prim-MSC produced equivalent platelet engraftment times [32]. Because there was only one RCT comparing BSC to Prim-MSC, it was deemed necessary to perform at least one more analysis.

The third round of analyses extended delta analyses to all comparisons. Utilizing delta engraftment times, studies were arranged by type of comparison and sorted by delta in ascending order. The results for granulocyte engraftment are presented in Table 1 and for platelet recovery in Table 2. As can be seen for granulocytes, 17 of 19 comparisons have a delta greater than 1 for BSC compared to SS-MSC. Also, 2 of 2 comparisons of Prim-MSC vs SS-MSC have delta...
greater than 1. Finally, 0 of 3 comparisons of BSC vs Prim-BSC have a delta greater than 0, let alone delta greater than 1. As can be seen for platelets, all 19 comparisons have a delta greater than 2 for BSC compared to SS-MSC. Also, 1 of 2 comparisons of Prim-MSC vs SS-MSC have a delta greater than 2. Finally, only 1 of 3 comparisons of BSC vs Prim-BSC has a delta greater than 2 (and that delta is 3).

Taking all of these three analyses together, it appears that allogeneic G-CSF Prim-MSC have the same engraftment potential as G-CSF-mobilized BSC. We believe that the functional capacity of Prim-MSC is the best measure of the repopulation potential of the marrow left behind after mobilization of BSC with G-CSF. Therefore, it appears that there should be only minimal concern about donor long-term hematopoiesis. Next, and high-GVID was less frequent with SS-MSC and Prim-MSC than with BSC but overall and disease-free survival were the same. Although chronic GVHD has a well documented graft-vs.-leukemia/lymphoma effect, the reduction of chronic GVHD seen with SS-MSC and Prim-MSC was not associated with an increase in relapse rates.

Factors influencing pace of engraftment

The literature review enabled us to collect data on a variety of clinical factors (from both autologous and allogeneic transplants) that may influence the pace of engraftment of granulocytes and platelets. Factors potentially relevant to the pace of engraftment included anatomic source of stem cells, use of G-CSF pretreatment prior to collecting stem cells from either source, dose of CD34+ cells/kg delivered to patient, use of G-CSF during the first 7 days after transplant, use of methotrexate as part of prophylaxis to prevent acute GVHD, diagnosis, and high-dose chemotherapy regimen. When median times to granulocyte and platelet recovery were plotted on a histogram, we noted that there was a normal distribution (as opposed to our nonparametric assumptions). This permitted us to use ANCOVA methodology to attempt to determine which of these factors, if any, may have had a significant influence upon the median day to recovery of granulocytes and platelets.

For autologous transplantation, the dose of CD34+ cells/kg is well known to be associated with time to engraftment for BSC [55]. The simplest fit mathematical equation describing this relationship is a hyperbolic function in which day of engraftment = constant + 1/(function of cell dose) [56]. Therefore, it was no surprise that, with a univariate analysis of median day to engraftment with CD34+ cell dose was performed, CD34+ cell dose was significantly related to the time of engraftment. However, quite surprisingly, when the use of G-CSF prior to collecting stem cells was entered into a bivariate analysis, CD34+ cell dose was no longer significantly associated with pace of engraftment. When additional variables were added to perform a full multivariate analysis, CD34+ cell dose was again not associated with time to engraftment (see Table 3). Reexamination of Figure 1 gives a graphic overview of this analysis because patients receiving Prim-MSC recovered granulocyte and platelet counts more rapidly than those receiving SS-MSC despite receiving one quarter fewer CD34+ cells (weighted median 1.5 x 10^9/kg vs 2.1 x 10^9/kg). The only factor that had a significant impact on engraftment was the use of G-CSF prior to collecting stem cells from either blood or marrow.

For allogeneic transplantation, the results of the multivariate analyses were very interesting as well. As shown in Table 3, for granulocyte recovery, CD34+ cell dose, the use of G-CSF during the first 7 days after engraftment, the use of methotrexate as part of prophylaxis for acute GVHD, and the stem cell product were all variables significantly and independently associated with pace of engraftment. CD34+ cell dose was important for granulocyte but not platelet recovery. The value of using G-CSF after engraftment has long been known for SS-MSC [57] but has been a subject for debate with BSC. Our analysis pointed to significant and independent role for G-CSF for granulocyte but not platelet recovery (as might be expected). Because use of methotrexate has long been known to delay allogeneic engraftment [55], it came as no surprise that methotrexate negatively but significantly influenced time to engraftment of granulocytes but, quite to our surprise, methotrexate had no impact upon engraftment of platelets. The interaction term told us that G-CSF pretreatment and anatomic source was responsible for accelerating engraftment by BSC and Prim-MSC of both granulocytes and platelets. Reexaming Figure 3, we see that Prim-MSC produced as rapid engraftment as BSC but with roughly one-third lower dose of CD34+ cells (4.1 x 10^9/kg vs 6.1 x 10^9/kg) and Prim-MSC engrafted much faster than SS-MSC with but one-third more CD34+ cells (4.1 x 10^9/kg vs 3.2 x 10^9/kg).

Identifying factors that may influence the kinetics of engraftment must be interpreted with a degree of caution because the studies were not designed to answer this question, nor is the analysis a true meta-analysis. However, these findings are provocative and may provide rationale and incentive for future prospective RCT.

Discussion

In the years 2001 and 2002, six publications appeared in peer-reviewed journals comparing engraftment kinetics of stem cells derived from peripheral blood and bone marrow in allogeneic transplants [27–29,46–48]. In these six publications, blood stem cells were observed to produce more rapid recovery of granulocytes and platelets than marrow stem cells. In all six reports, BSC were mobilized by G-CSF but MSC were collected in the steady state. These data supported the hypothesis that BSC engraft more rapidly than MSC, a hypothesis that was based upon at least 13 publications in the allogeneic literature through the year 2000 [33–45].
Table 3. Multivariate analyses for clinical factors influencing pace of engraftment

<table>
<thead>
<tr>
<th>Clinical Factor</th>
<th>Favorable Variable</th>
<th>Two-tailed p for Median Days to</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous transplants</td>
<td>CD34+ Cell Dose (× 10^6/kg)</td>
<td>more</td>
<td>0.003</td>
</tr>
<tr>
<td>Use of G-CSF during First 7 Days Protransplant</td>
<td>yes</td>
<td></td>
<td>0.660</td>
</tr>
<tr>
<td>Characteristics of Stem Cell Product</td>
<td></td>
<td>BSC</td>
<td>0.012</td>
</tr>
<tr>
<td>1. Anatomic Source of Stem Cells</td>
<td></td>
<td>BSC</td>
<td>0.031</td>
</tr>
<tr>
<td>2. Precollection Use of G-CSF</td>
<td></td>
<td>not SS-MSC</td>
<td>0.050</td>
</tr>
<tr>
<td>Allogeneic Transplants</td>
<td>CD34+ Cell Dose (× 10^6/kg)</td>
<td>more</td>
<td>0.012</td>
</tr>
<tr>
<td>Use of G-CSF during First 7 Days Posttransplant</td>
<td>yes</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Use of Methotrexate as Part of Acute GVHD Prevention</td>
<td>no</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>Characteristics of Stem Cell Product</td>
<td></td>
<td>BSC</td>
<td>0.395</td>
</tr>
<tr>
<td>1. Anatomic Source of Stem Cells</td>
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<tr>
<td>2. Precollection Use of G-CSF</td>
<td></td>
<td>not SS-MSC</td>
<td>0.050</td>
</tr>
</tbody>
</table>

However, there are now five reports in the literature [30–32,49,50] evaluating the engraftment kinetics for Prim-MSC. In two of these five reports, G-CSF Prim-MSC have been shown to engraft more rapidly than SS-MSC. In three of these five reports, G-CSF Prim-MSC engrafted as rapidly as did G-CSF BSC. These five reports suggest that the hypothesis that BSC engraft more rapidly than MSC needs reexamination if not reformulation.

Several reports have appeared in the literature in 2001 and 2002 demonstrating that G-CSF induces neutrophil precursors in the marrow microenvironment that cleave VCAM-1 (CD106), an event that appears to be mechanistically related to the release of hematopoietic progenitors into the circulation. Clinically, this is termed "mobilization" [10–13]. It has also been shown that chemotherapy induces the same proteolytic environment [11]. The proteolytic environment may produce damage to stem cells remaining resident in the marrow or damage to the marrow stroma after routine use of chemotherapy (with or without G-CSF). The consequences of the proteolytic environment are not a critical factor in patients with malignancies, as chemotherapy is necessary to treat their malignancies. However, this proteolytic environment may possibly be one of the explanations why up to 20% of eligible autologous transplant patients cannot have sufficient numbers (qualitatively as well as quantitatively) of stem cells mobilized into the bloodstream to ensure rapid engraftment after high-dose therapy. More importantly, the potential that this proteolytic environment may produce permanent stem cell damage in healthy donors is an issue that needed to be addressed, especially now that unrelated volunteer donors are being mobilized with G-CSF to collect BSC [9]. Gratifyingly, this review shows that this proteolysis does not appear to damage the marrow in a manner that would be detrimental to a healthy donor (e.g., damage to the microenvironment or depletion of stem cells) because Prim-MSC, the very MSC remaining after G-CSF treatment to mobilize and collect BSC, retain their early engraftment potential. The effects of this proteolysis upon the marrow microenvironment remain to be determined.

The literature review examining the engraftment of G-CSF Prim-MSC allows us to achieve a better understanding of the results of early studies about BSC and MSC that, today, seem to be in contradistinction to the prevailing hypothesis that BSC engraft more rapidly than MSC. First, granulocyte recovery after autologous stem cell transplantation was very slow when steady-state BSC (SS-BSC) were employed (median day 22 (11–58)) [7] as compared to when autologous chemotherapy and G-CSF-mobilized BSC were employed (median day 10 (8–52)) [55]. Second, autologous SS-BSC produced apparently slower granulocyte recovery [7] than did allogeneic SS-MSC (median day 14 (10–37)) when methotrexate was not used along with cyclosporine for acute GVHD prophylaxis [55]. And third, in an RCT, there was no acceleration of granulocyte engraftment when autologous SS-BSC were added to autologous SS-MSC as compared to autologous SS-MSC alone (medians 27 vs 20 days) for granulocyte recovery [58]. Data concerning platelet recovery show the same relationships: autologous SS-BSC, median day 23 (14–56); autologous chemotherapy and G-CSF BSC, median day 17 (8–75); and allogeneic SS-MSC,
median day 25 (11–81). Finally, for platelet recovery, autologous SS-BSA combined with autologous SS-MSA did not engraft more rapidly than autologous SS-MSA alone (medians 27 vs 22 days) [58]. Altogether these data suggest that, in terms of engraftment, steady-state BSC have no better engraftment potential than steady-state MSC.

If, as described above, SS-BSA and SS-MSA have equivalent engraftment potential and if, as summarized by this review, Prim-MSA and mobilized BSC have equivalent engraftment potential, then we must develop a new hypothesis about engraftment; to wit, MSC and BSC have equivalent engraftment potential and G-CSF enhances the engraftment potential of both MSC and BSC equally as well. This G-CSF-induced acceleration of engraftment is seen when G-CSF pretreatment is identical before leukapheresis for BSC and before marrow harvest for Prim-MSA.

Autologous transplantation is not the best model system for verifying the validity of this new hypothesis because of the tremendous heterogeneity introduced in the engraftment potential of collected stem cells due to prior chemotherapy and the effects of different transplant preparative regimens upon engraftment [55, 57, 59, 60]. Indeed, at least one group of investigators did not find that G-CSF priming accelerated engraftment of MSC at all [17]. However, this one negative observation cannot outweigh the several positive observations [61]. More importantly, we have previously reported acceleration of engraftment by G-CSF priming of autologous MSC [62] and carried this observation through an RCT, which demonstrated equivalent engraftment for both BSC and Prim-MSA [24]. The argument could be proffered that there are an insufficient number of autologous RCT to support the new hypothesis. The principles of evidence-based medicine hold that RCT (phase II trials) are considered the gold standard in clinical research. However, there are new data that reveal non-RCT (phase II trials) establish biological principles with the same durability of validity as do RCTs [63]. This provides us with an entirely new perspective for examining the published literature.

Allogeneic transplantation is a far better human model because health of donor marrow is not in question as it is in patients with malignancies. In comparative studies, many more allogeneic transplants involving BSC (n = 1949), SS-MSA (n = 3782), and G-CSF Prim-MSA (n = 107) have been reported than autologous transplants involving the same three groups (BSC, 407; SS-MSA, 347; and Prim-MSA, 159). The numbers of Prim-MSA transplants are few but the data are clear. In the global analysis, in the examination of RCT only, and in delta analysis comparisons of all studies, the allogeneic data support the new hypothesis that stem cell engraftment potential is not predetermined by the anatomic site of collection but rather by the effects of G-CSF upon the collected cells.

The new hypothesis we postulate may be confirmed by RCTs in which the following parameters are held constant for all patients in the study: only one age group, a single diagnosis and state of disease (preferably first complete remission for the acute myelogenous leukemia to minimize induction chemotherapy variability), a limited interval between demonstration of remission and transplant, a fixed G-CSF pretreatment regimen, standardized collection of stem cells by leukaphereses and by bone marrow harvest, one transplant preparative regimen, infusion of a specified and equal number of CD34+ cells for both BSC and MSC, a single anti-GVHD immunosuppressive regimen (preferably without methotrexate), and one policy concerning use of G-CSF postgrafting. This is a tall order and, to be accomplished, would likely require a large number of institutions and probably international collaboration. The number of institutions required adds additional variability, but the variability may be manageable by stratification by institution. The likelihood that this will be done in the near future is poor. In the meantime, clinicians need to decide care for their patients with the data at hand. This brings up other observations about outcomes that can be addressed.

Contamination of stem cell collection could be a determinant in choosing the anatomic source of stem cells (BSC vs MSC) for autologous transplantation. But this does not appear to be a major consideration, at least for lymphomas [64]. Healthy donor anxiety and donor choice could be a factor in determining the source of stem cells were there not other important clinical considerations for the patient receiving allogeneic transplantation (vide infra) [65].

For autologous transplantation, relapse rate, overall survival, and disease-free survival are very important clinical outcomes. A recent publication concerning patients with non-Hodgkin's lymphoma (NHL) only, transplanted after a single preparative regimen, showed an overall survival benefit for BSC over SS-MSA but no disease-free survival advantage [14]. Four other RCT, involving germ cell cancer (1), NHL and Hodgkin's disease combined (2), and breast cancer (1), treated by four different high-dose regimens show no disease-free survival advantage for BSC over SS-MSA [16, 23, 24, 26]. Moreover, none of these four RCT shows an overall survival advantage for BSC over SS-MSA. The sentinel article [14] is a perfect demonstration that overall survival is not the best measure of value of a therapy, especially if there are good salvage therapies to prolong life after relapse [53]. As limited as the global analysis may be, there does not appear to be a consistent advantage of BSC over SS-MSA insofar as survival and disease-free survival are concerned. This is all the more meaningful when considering that the median follow-up after autologous transplantation for overall survival was 3.6 years and 2.8 years for disease-free survival.

In allogeneic transplantation, acute and chronic GVHD, in addition to relapse rate, overall survival, and disease-free survival, are very important clinical outcomes. No comparative engraftment study reported so far had primary endpoints of either GVHD, relapse, or survival. Evaluations of secondary endpoints have resulted in reports showing advantage
for SS-MSC over BSC in terms of reduced incidence of chronic GVHD [27,32,54,66]. Our global analysis supports this observation. The mechanism for this effect may be the nearly one log fewer CD34+ cells/kg in SS-MSC compared to BSC and/or vastly fewer regulatory NK-1 cells and/or CD4+ /CD25+ regulatory T cells in BSC compared to SS-MSC, leading to reduction in GVHD potential of marrow T cells [67,68]. Furthermore, pretreatment of the donor with G-CSF may also reduce the potential of Prim-MSC to produce GVHD vis-à-vis SS-MSC [69]. With respect to relapse and survival outcomes, two large RCT suggested that overall survival was improved for recipients of BSC compared to SS-MSC [27,28]. However, looking at all nine of the prospective allogeneic RCT since 1998, there are four (including the two above) that show better overall or disease-free survival results for BSC and five that show equivalence of SS-MSC (n = 2) or better results for SS-MSC (n = 2) or Prim-MSC (n = 1). Moreover, a multicenter RCT comparing CD34+ cells selected from BSC and SS-MSC demonstrated improved survival for patients with hematologic malignancies and myelodysplasia receiving CD34+ cells selected from SS-MSC [70]. Our global analysis reflects this variability in observations by showing no particular overall or disease-free survival for BSC over SS-MSC.

On the one hand, although Prim-MSC appear to have at least equivalent if not better outcomes when compared to BSC, there are too few Prim-MSC patients to draw definitive conclusions at this time. On the other hand, taking all of the observations about GVHD, relapse, and survival together, it would appear that selecting Prim-MSC as the stem cell product may be appropriate because of less chronic GVHD. Less chronic GVHD could mean less graft-vs-malignancy effect, which may translate into poorer overall survival [27,28]. Resolving these issues is why carefully performed studies are required to determine the balance. Increasing the number of patients in the trial we proposed above would improve the power to detect differences in GVHD incidence, relapse rates, and survival outcomes as well.

G-CSF does mobilize stem cells from the marrow, most likely via proteolysis [10–13], but both BSC and the residual MSC (i.e., Prim-MSC) have the same engraftment potential. It appears that BSC collected in the steady state have the same engraftment potential as SS-MSC. Finally, SS-BSC and SS-MSC engraft more slowly than Prim-MSC and BSC. This difference in engraftment kinetics cannot be explained by differences in CD34+ cell doses infused. In fact, Prim-MSC contain on average 30% fewer CD34+ cells/kg than SS-MSC in autologous transplants and only 20% more CD34+ cells in allogeneic transplant. Thus, there must be a “second effect” of G-CSF upon stem cells that enables them to produce more rapid early engraftment.

The “second effect” may very well be, in man as it is in mice, differences in integrin expression between BSC and residual Prim-MSC that were produced by mobilization treatment [71]. Cyclophosphamide and G-CSF induce downregulation of a2 integrin and upregulation of a5 integrin expression in Prim-MSC in mice. BSC bear significantly lower levels than Prim-MSC of several integrins. These differences are associated with a 50% reduction in marrow homing potential of BSC vis-à-vis Prim-MSC in the mouse model. In addition, human BSC grafts contain 50 to 60% more CD34+ cells than G-CSF Prim-MSC. Equal engraftment rates for BSC and Prim-MSC products suggest higher engraftment efficiency for Prim-MSC. Moreover, autologous G-CSF Prim-MSC, collected after failure to mobilize BSC, engrafted rapidly (day +12 for granulocytes and day +13 for platelets) [72]. Finally, the “second effect” may be deciphered from the differential expression of at least 27 genes involved in cell cycle, DNA synthesis, cell-cycle initiation, proapoptosis, and receptors [73].

To conclude, the answer to the original question posed in the abstract of this review is Prim-MSC engraft as rapidly as BSC because of G-CSF treatment before collection. The anatomic site of stem cell acquisition is not the determining factor for pace of engraftment. With the data we have summarized, use of Prim-MSC seems acceptable at the present time. However, we would like to see Prim-MSC studied in direct comparison with BSC.

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Progenitor content of autologous grafts: mobilized bone marrow vs mobilized blood

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Summary:

The progenitor content of autologous peripheral blood progenitor and stem cell collections is a major determinant of prompt hematopoietic recovery following autologous stem cell transplantation. We analyzed unstimulated bone marrow (BM) and peripheral blood (PB) apheresis products in comparison to those collected following G-CSF or GM-CSF stimulation. We quantitated their committed (CFU-GM) and primitive (long-term culture-initiating cells, LTC-IC) progenitors in relation to hematologic recovery in 63 patients undergoing autografting for lymphoid malignancies. G-CSF, but not GM-CSF, substantially enriched the committed progenitor content (2.5-3.6-fold) of both PB and BM grafts. G-CSF also enriched the LTC-IC content of BM and PB compared to control grafts. GM-CSF augmented (11.5-fold) the LTC-IC content of stimulated BM, but not GM-CSF-mobilized PB. Neutrophil recovery was substantially quicker in recipients of BM or PB mobilized with G-CSF or GM-CSF. In contrast, red cell and platelet recovery was accelerated in recipients of GM-CSF-stimulated BM (but not PB) and G-CSF-stimulated PB (but not BM). No direct correlation between progenitor dose and hematopoietic recovery for neutrophils, platelets or red cells was observed. Cytokine stimulation can augment the committed and more primitive multilineage progenitor content of BM and PB grafts, to a differing extent. The uncertain relationship with multilineage myeloid recovery emphasizes the limitations in using clonogenic progenitor analyses to assess the adequacy of an autologous graft prior to transplantation.

doi:10.1038/sj.bmt.1704237
Keywords: hematopoietic progenitors; autologous graft; cytokine mobilization

Cytokine mobilization has been widely used over a decade, most often to collect peripheral blood stem cells (PBSC) as a cellular product used for hematopoietic support during autologous stem cell transplantation. In contrast, cytokine-stimulated bone marrow stem cell (BMSC) autografts are rarely used. We investigated the committed and primitive progenitor content of autologous BMSC and PBSC grafts from patients with malignant non-Hodgkin’s lymphoma (NHL) and Hodgkin’s disease (HD) in order to understand the effects of cytokine stimulation on the BM or PB progenitor content and their relation to post transplant hematologic recovery.

Patients and methods

Patient characteristics

As shown (Table 1), all patients were recipients of autologous hematopoietic stem cell grafts at the University of Minnesota. All but three of the 63 patients were adults and approximately 85% had NHL, with the remaining 15% having HD. A total of 25 patients had unimpaired, unstimulated collections: 15 BM harvest and 10 PBSC apheresis. In all, 38 had cytokine-stimulated collections: 20 with G-CSF and 18 with GM-CSF as described. Patients’ age, gender and primary diagnosis were similar in the six cohorts, those receiving BMSC or PBSC, either control, G-CSF- or GM-CSF-stimulated grafts.

Graft collection and mobilization

BM aspiration harvests were performed under general anesthesia by small volume (5–10 cm³) aspirates from bilateral iliac crest sites to a target collection of 1.5 L. PBSC were collected by continuous flow apheresis using the Baxter–Fenwall CS3000® Plus set up for stem cell collection with a small volume collection chamber. Unstimulated control PBSC were collected in four daily consecutive aphereses processing 10–121 of blood per day. Cytokine mobilization prior to collection used either GM-CSF (250 μg/m²/day) injected subcutaneously round to vial size; for example, 250 μg/day for patients <1.2 m² or 500 μg for patients over 1.2 m²) or G-CSF (250 μg/m²/day rounded to vial size: 300 or 480 μg/day). Stimulated BM harvests were collected on day 6 after 5 days of cytokine. Stimulated PBSC were collected on days 6–9 of daily cytokine therapy. Cytokines used for mobilization were assigned in random fashion. Patients studied represented a
subset of those participating in two trials comparing BMSC to PBSC as control unstimulated or cytokine-mobilized samples.10,11

Transplants were performed as previously reported following preconditioning with cyclophosphamide and total body irradiation (TBI) for patients with NHL or cyclophosphamide, Carmustine and etoposide for HD or for NHL patients ineligible to receive TBI safely.10,11 All previously collected and cryopreserved grafts were infused on day 0 and post transplant all patients received daily G-CSF (5 μg/kg/day rounded to vial size) until neutrophil recovery > 2.5 x 10^9/L for 2 consecutive days. All treatment protocols were reviewed and approved by the University of Minnesota Institutional Review Board and all subjects exercised written informed consent prior to treatment.

**In vitro progenitor culture**

Aliquots of BM harvests or PBSC apheresis collections were cryopreserved in 10% human serum and 10% DMSO using controlled rate-freezing techniques and stored in the liquid phase of liquid nitrogen. The transplants were performed in separate cohorts under different treatment protocols.10,11 All cryopreserved samples were thawed and plated in one series of culture studies using consistent techniques. Aliquots were thawed, washed in sterile media and resuspended in Iscove's modified Dulbecco's medium (IMDM). Committed progenitors (CFU-GM) were cultured in methylcellulose containing IMDM supplemented with 30% fetal calf serum with erythropoietin (EPO, 31U/ml) and 10% supernatant of bladder carcinoma cell line, 5637. After 14 days culture at 37°C in humidified 5% CO2, myeloid colonies (CFU-GM) were counted using an inverted microscope and calculated in relation to the plated cellular inoculum.13 More primitive progenitors, long-term culture-initiating cells (LTC-IC), were determined by plating thawed washed mononuclear cells on irradiated allogeneic BM stroma in IMDM supplemented with 12.5% fetal calf serum, 12.5% horse serum, 2 mmol/l glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 10^-4 mol/l hydrocortisone. After 5 weeks, all media were removed and overlaid with methylcellulose containing media supplemented with EPO and 5637 supernatant. After 14 additional days of culture, the presence of secondary CFU-GM was scored.12 The progenitor frequency within the BM or PBSC grafts allowed calculation of the graft progenitor content in relation to the nucleated cell dose infused/kg recipient weight. CD34 determinations on the graft aliquots were not routinely performed and not available for analysis.

**Statistical analysis**

Clinical data were collected and monitored by the University of Minnesota Bone Marrow Transplant Database and Biostatistical Support Facility that contains prospectively collected data on all individuals transplanted at our center. Comparative analysis of progenitor content in grafts from different patient cohorts was performed using the general Wilcoxon's test to compare CFU-GM/kg and LTC-IC/kg between groups. For comparisons involving the four collections of PBSC, the mean yield of each patient's PBSC collection was multiplied by four. The ratio of patient group medians was used to estimate the fold increase in progenitors over the unstimulated control groups. Confidence intervals of 95% were then generated around this statistic using bootstrap samples.

Hematopoietic engraftment was defined as the first of three serial post transplant measures of an absolute neutrophil count (ANC) > 5 x 10^9/L, an untransfused platelet count > 20 x 10^9/L and an untransfused hemoglobin > 8.0 g/dl. Correlation analysis of engraftment end points and the progenitor dose infused was performed using Spearman's rank-correlation coefficients. Patients who died before day +42 (n=2) without stable engraftment were excluded when calculating correlation estimates. Survivors beyond day +21 with failure of recovery in 1 or more lineages (n=3) were included in calculations and were assigned the highest rank of time to engraftment. Given that there were numerous comparisons of progenitor content and multilineage recovery, as a correction for
multiple testing, a P-value < 0.01 was used to indicate statistical significance for these clinical correlations. Threshold doses were sought to determine the lowest dose at which the majority of subjects reached hematopoietic recovery at standard times.

Results

Cytokine mobilization of primitive and committed hematopoietic progenitors

Mobilized grafts compared to controls. The progenitor content within the BM and PBSC grafts is shown in Table 2. Cytokine-mobilized BM collections using either G-CSF or GM-CSF contained significantly (11-29-fold) more primitive progenitors (LTC-IC/kg) than resting BM harvests (Table 2b, P = 0.05). Somewhat greater LTC-IC content of G-CSF-stimulated BM was apparent compared to GM-CSF-stimulated BM (P = 0.07). Analysis of the committed progenitor content of BM grafts showed that G-CSF, but not GM-CSF pretreatment, led to significant, 2.5-fold augmentation of the committed progenitor content (CFU-GM/kg) over control, unstimulated BM grafts.

Cytokine stimulation augmented the progenitor content of the PBSC grafts to a lesser extent than that observed in BM. PB grafts mobilized with G-CSF contained 3.6-fold more CFU-GM/kg and 2.6-fold more LTC-IC/kg compared to unstimulated PBSC. In contrast, GM-CSF-mobilized PBSC contained only 0.9-fold more CFU-GM/kg and 1.2-fold more LTC-IC/kg than control PB grafts.

G-CSF stimulated the CFU-GM content of both BM and PBSC grafts to a similar extent. As shown in Table 2, G-CSF-stimulated BM contained a median 2.5-fold increased content of CFU-GM over control BM, while G-CSF-stimulated PB had a 3.6-fold increase over control PBSC. However, G-CSF led to a 28.7-fold median increase in BM LTC-IC/kg, although only a small and nonsignificant increment in PBSC LTC-IC. In contrast, GM-CSF significantly enhanced the LTC-IC content (11.5-fold) over control BM, but did not increase the CFU-GM content of BM (1.1-fold).

PBSC grafts compared to BM. PBSC grafts contained a mean of 1.87-fold more nucleated cells/graft compared to BMSC. As shown in Table 2a, unstimulated PBSC grafts contained 12.8-fold more LTC-IC/kg than control BM grafts, while those primed with G-CSF and GM-CSF contained only 1.17- to 1.35-fold more LTC-IC/kg than BM grafts, respectively. The committed progenitor contents of PBSC and BMSC grafts were similar. PBSC contained 1.06-, 1.51- and 0.85-fold more CFU-GM/kg than BM grafts in control, G-CSF and GM-CSF primed grafts, respectively.

Hematologic recovery using cytokine-mobilized grafts

In serial patient cohorts, autotransplantation was performed using control grafts and using grafts collected after cytokine priming of BM and PB using either G-CSF or GM-CSF. Transplantation was followed by neutrophil engraftment (ANC > 5 x 10^9/L) at a median of 13-16 days in the cytokine-mobilized cohorts compared to a median of 22 days in those receiving unstimulated BM or PB grafts (Table 3). Lister, more variable recovery of platelet and RBC production was observed. As shown, GM-CSF stimulation prior to BM harvests led to platelet and RBC engraftment at a median of 18 and 14 days, respectively, compared to a median of 25 and 20 days in the unstimulated BM controls. G-CSF stimulation of BM grafts yielded platelet and RBC recovery at a median of 27 days.

Transplantation using PBSC led to faster hematologic recovery. Grafts with G-CSF-mobilized PBSC were followed by engraftment of platelets and RBC at a median of 13 and 14 days, respectively. GM-CSF-mobilized PBSC did

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Progenitor content (CFU-GM and LTC-IC) in control and cytokine-mobilized autologous grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>(a) Progenitor content</td>
<td></td>
</tr>
<tr>
<td>BM: G-CSF</td>
<td>10</td>
</tr>
<tr>
<td>BM: GM-CSF</td>
<td>7</td>
</tr>
<tr>
<td>BM: control</td>
<td>15</td>
</tr>
<tr>
<td>PBSC: G-CSF</td>
<td>10</td>
</tr>
<tr>
<td>PBSC: GM-CSF</td>
<td>11</td>
</tr>
<tr>
<td>PBSC: control</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CFU-GM/kg fold increase (95% CI)</th>
<th>LTC-IC/kg fold increase (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM: G-CSF</td>
<td>2.5 (1.2-5.1)*</td>
</tr>
<tr>
<td>BM: GM-CSF</td>
<td>1.1 (0.4-3.6)</td>
</tr>
<tr>
<td>PBSC: G-CSF</td>
<td>3.6 (0.4-8.1)</td>
</tr>
<tr>
<td>PBSC: GM-CSF</td>
<td>0.9 (0.1-2.6)</td>
</tr>
</tbody>
</table>

*Progenitors/kg (x 10^9) determined from in vitro culture of autologous hematopoietic grafts are shown. *P = 0.05 vs unstimulated control (same cell source); **P = 0.07 vs GM-CSF-stimulated grafts (same cell source); ***P = 0.05 vs GM-CSF-stimulated grafts (same cell source); *P = 0.03 vs BM control.

The ratios of group medians of progenitor content of cytokine-stimulated BM and PBSC grafts are shown; fold increase over control. *P = 0.05.

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not lead to accelerated hemophagocytosis with time to platelet recovery at median day 42 and RBC day 38, similar to control PBSC transplant platelet and RBC recovery at 38 and 27 days, respectively. Thus, G-CSF or GM-CSF mobilization of either BM or PB was associated with rapid neutrophil recovery. In contrast, GM-CSF, but not G-CSF stimulation of BM grafts led to somewhat more rapid platelet and RBC recovery. G-CSF, but not GM-CSF, mobilization of PB grafts accelerated both platelet and RBC recovery.

**Graft progenitor content in relation to multilineage engraftment**

We analyzed the infused dose of primitive and committed progenitors as potential predictors of the speed of post transplant engraftment. As shown in Table 4, we observed no direct correlation between the infused progenitor dose (CFU/kg) and the speed of hematopoietic reconstitution of neutrophils, RBCs or platelets. No such correlation was observed in either G-CSF- or GM-CSF-stimulated BM or PBSC. Similarly, the infused dose of LTC-IC/kg did not correlate with the speed of multilineage engraftment following either GM-CSF- or G-CSF-stimulated BM or PBSC transplantation.

We also sought evidence for a threshold progenitor dose, above which prompt myeloid engraftment was assured. Disappointingly, we found no such threshold (data not shown). We also investigated a possible relationship between a minimum infused progenitor dose and subsequent delayed platelet or RBC recovery, but again found no such correlation.

**Discussion**

G-CSF and GM-CSF have a distinct functional impact on both the proliferation and translocation of primitive and committed progenitors into the blood and marrow. While either cytokine may accelerate hematopoietic recovery after myelosuppressive therapy or stem cell transplantation, their comparative effect on the progenitor content of the autologous hematopoietic grafts is less certain. In this study, we compared both cytokine-stimulated and control grafts collected from BM and PB. We observed that cytokine stimulation using either G-CSF or GM-CSF was more successful at mobilizing or expanding the number of more primitive stem cells (LTC-IC) in BM, but not in PB. Whereas G-CSF and GM-CSF have been shown to enhance the cell cycling fraction of CFU-GM in BM, there has been only limited study of cytokine mobilization techniques on LTC-IC expansion in BM-derived LTC-IC compared to those found in cytokine-stimulated PB.17

Our observation that cytokine stimulation produced more LTC-IC in BM compared to PB may suggest an intrinsic difference in LTC-IC subsets and their response to cytokines depending on location.13 In BM progenitors, expansion without translocation is necessary, while in PB, expansion must be accompanied by translocation through altered adhesive interaction with BM stroma. In BM, G-CSF pretreatment was more effective than GM-CSF in augmenting the final BM graft content of LTC-IC. G-CSF was more effective than GM-CSF in mobilization of progenitors into PBSC, but for this compartment, CFU-GM were most effectively mobilized. Previous study by Ho

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**Table 3** Multilineage hematologic engraftment in cohorts infused with control- or cytokine-mobilized autografts

<table>
<thead>
<tr>
<th>Group (N)</th>
<th>Days to neutrophil engraftment</th>
<th>Days to platelet independence</th>
<th>Days to RBC independence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM: G-CSF (10)</td>
<td>14 (10-23)</td>
<td>27 (9-45)</td>
<td>27 (7-59)</td>
</tr>
<tr>
<td>BM: GM-CSF (17)</td>
<td>13 (11-14)</td>
<td>18 (12-30)</td>
<td>14 (10-35)</td>
</tr>
<tr>
<td>BM: control (15)</td>
<td>22 (11-44)</td>
<td>25 (17-51)</td>
<td>20 (11-63)</td>
</tr>
<tr>
<td>PBSC: G-CSF (10)</td>
<td>14 (11-15)</td>
<td>13 (9-33)</td>
<td>14 (8-39)</td>
</tr>
<tr>
<td>PBSC: GM-CSF (11)</td>
<td>16 (12-52)</td>
<td>42 (11-191)</td>
<td>38 (4-170)</td>
</tr>
<tr>
<td>PBSC: control (10)</td>
<td>22 (11-44)</td>
<td>38 (14-93)</td>
<td>27 (10-76)</td>
</tr>
</tbody>
</table>

Median days (range) to recovery for neutrophil, platelet and RBC recovery after autologous stem cell transplantation are shown. Patients dying prior to day +21 (without recovery) were excluded, but later deaths without single lineage (n = 2) or multilineage recovery (n = 1) were analyzed as graft failure (total n = 3).

**Table 4** Correlation between progenitor dose and hematopoietic recovery post autotransplantation

<table>
<thead>
<tr>
<th></th>
<th>Neutrophil engraftment R (P-value)</th>
<th>Platelet independence R (P-value)</th>
<th>RBC independence R (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CFU-GM</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM: G-CSF</td>
<td>0.48 (0.16)</td>
<td>0.05 (0.8)</td>
<td>0.24 (0.51)</td>
</tr>
<tr>
<td>BM: GM-CSF</td>
<td>0.74 (0.10)</td>
<td>0.25 (0.39)</td>
<td>0.35 (0.43)</td>
</tr>
<tr>
<td>BM: control</td>
<td>0.38 (0.16)</td>
<td>0.32 (0.34)</td>
<td>0.03 (0.8)</td>
</tr>
<tr>
<td>PBSC: G-CSF</td>
<td>0.51 (0.13)</td>
<td>0.58 (0.06)</td>
<td>0.46 (0.19)</td>
</tr>
<tr>
<td>PBSC: GM-CSF</td>
<td>0.59 (0.06)</td>
<td>0.36 (0.28)</td>
<td>0.38 (0.25)</td>
</tr>
<tr>
<td>PBSC: control</td>
<td>0.08 (0.8)</td>
<td>0.47 (0.24)</td>
<td>0.29 (0.49)</td>
</tr>
<tr>
<td><strong>LTC-IC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BM: G-CSF</td>
<td>0.12 (0.78)</td>
<td>0</td>
<td>0.17 (0.69)</td>
</tr>
<tr>
<td>BM: GM-CSF</td>
<td>0.09 (0.68)</td>
<td>0.63 (0.09)</td>
<td>0.14 (0.76)</td>
</tr>
<tr>
<td>BM: control</td>
<td>0.16 (0.71)</td>
<td>0.26 (0.62)</td>
<td>0.26 (0.62)</td>
</tr>
<tr>
<td>PBSC: G-CSF</td>
<td>0.40 (0.28)</td>
<td>0.45 (0.23)</td>
<td>0.46 (0.19)</td>
</tr>
<tr>
<td>PBSC: GM-CSF</td>
<td>0.24 (0.47)</td>
<td>0.30 (0.37)</td>
<td>0.38 (0.25)</td>
</tr>
<tr>
<td>PBSC: control</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
</tbody>
</table>

The Spearman rank-correlation coefficients (P-value) between progenitor dose infused/kg (CFU-GM and LTC-IC) and lineage-specific hematologic recovery following autotransplantation are shown. NE = not evaluable (n < 5).
et al. showed G-CSF stimulation to be more effective than GM-CSF at mobilizing CFU-GM into PB. It is interesting that G-CSF increases the mobilization of more primitive progenitors into BM, but yields more committed progenitors in PB.

More rapid recovery of hematopoiesis can reduce mortality as well as the morbidity and cost of autologous transplantation. Current practice in graft evaluation to predict post transplant hematopoietic recovery is contingent on a sufficient CD34+ transplant dose, where a dose of $\geq 5 \times 10^8$ CD34+ cells/kg delivers consistently rapid hematopoietic recovery. Previous studies have shown that using PB autografts containing $\geq 5 \times 10^8$ CD34+ cells, neutrophil and platelet recovery within 9–12 days can be expected.

We observed modest differences in time to engraftment when comparing grafts mobilized with either G-CSF or GM-CSF. Transplants using GM-CSF-stimulated BM and G-CSF-stimulated PB showing earlier engraftment are compatible with the hypothesis, suggesting enrichment of marrow progenitors by GM-CSF and increased translocation of progenitors by G-CSF. Combination studies of cytokines along with inhibitors of progenitor adhesion could enhance this engraftment even further, although most such investigations have focused on preclinical models.

We investigated a relationship between either CFU-GM or LTC-IC dose as more accurate predictors of the time to engraftment. Although we observed that G-CSF and GM-CSF have differing capacity for LTC-IC and CFU-GM enhancement in BM or PB, the progenitor dose infused did not correlate with time to hematopoietic reconstitution. In fact, correlation coefficients across all groups did not suggest any trend supporting a biologic association with the engraftment end points. It is acknowledged that limited sample size and possible heterogeneity in prior chemotherapy and other clinical factors may limit the power of our analysis.

Sutherland et al. also found no correlation between the number of LTC-IC in PB with hematopoietic reconstitution. Previous studies have suggested a strong correlation between CFU-GM dose and hematopoietic reconstitution, although we observed neither a direct correlation nor a threshold dose of LTC-IC or CFU-GM predictive of hematopoietic reconstitution. Unavailability of CD34 graft content also compromised our study. Recently, Hogge et al. reported that 90% of patients became platelet transfusion independent by 28 days posttransplant if their grafts contained either $\geq 2 \times 10^8$ CD34+ cells/kg, $\geq 5 \times 10^5$ total colony-forming cells (CFCs)/kg, or $\geq 1000$ CFC in 5-week LTC-IC/kg. Quantitation of phenotypic (CD34+, CD34+ 38-) or clonogenic (cobblestone area-forming cells) progenitors can possibly define an inadequate graft, especially after intensive prior alkylator-based chemotherapy. Further study will be required for more reliable assessments of the quality of hematopoietic cells within a cryopreserved autograft. Recent interest in cytokine-stimulated BM allografts as well as widespread application of G-CSF-mobilized PB allografts further suggests the need for careful quantitative, as well as qualitative characterization of graft content.

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INTRODUCTION

Although hematopoietic cell transplantation (HCT) has the potential to cure high-risk hematologic and nonhematologic disorders, it is a complex, resource-intensive, and costly procedure. Costs of transplantation can include charges for delivery of care (eg, physician charges), supportive care (eg, blood product transfusions), graft procurement, hospitalization, pharmacy, and laboratory and radiologic investigations. Also, despite major advances in transplant techniques and supportive care practices, HCT continues to be associated with substantial treatment-related mortality (TRM) such as infections, organ failure, and graft-versus-host disease (GVHD), and management of these complications can further increase the overall cost of posttransplant care. Studies of transplantation costs are complex and difficult to conduct because of the wide variation in transplant methods, conditioning, and GVHD prophylaxis regimens and supportive care practices. However, studies have described costs of allogeneic HCT using either myeloablative or nonmyeloablative conditioning regimens, and show that HCT in general is an
expensive procedure, and occurrence of complications after transplantation increases overall medical costs [1-10].

Introduction of unrelated umbilical cord blood (UCB) as an alternative graft source for patients without a matched related donor (MRD) is among the most significant recent breakthroughs in the field of transplantation. UCB has the advantages of rapid availability, and may be associated with lower risks of GVHD, despite the use of units with higher HLA disparity [11,12]. Our group has described the use of UCB HCT in adults, especially using 2 UCB units to optimize cell dose with outcomes comparable to that seen with other donor sources [13-15]. However, use of UCB is associated with delayed engraftment and a higher risk of graft failure. Incremental experience is rapidly leading to adoption of UCB as an alternative donor source by many transplant centers. A better understanding of the costs of UCB HCT is of importance from the health care resource utilization and health policy perspective, and can also assist in comparison of cost-effectiveness of UCB with other alternative (eg, matched unrelated donor and haploidentical) donor sources. Identification of specific factors that may be predictive for UCB transplant costs can help with the development of strategies to reduce costs while maintaining outcomes with a resultant increase in the applicability of UCB HCT.

We conducted a retrospective cohort study in a contemporary group of adult HCT recipients to evaluate the costs of myeloablative and nonmyeloablative UCB HCT, and to compare them with the costs of myeloablative and nonmyeloablative MRD HCT. We also explored various risk factors for their association with increased costs of HCT.

PATIENTS AND METHODS

Patients

The study cohort consisted of consecutive patients who received an allogeneic HCT between 2004 and 2006, and were ≥18 years of age at the time of transplantation. From the 318 eligible patients, 24 were excluded: recipients of planned autologous HCT followed by nonmyeloablative allogeneic sibling donor HCT for multiple myeloma (MM; N = 12), and recipients of matched unrelated donor grafts (N = 12). Hence, the final study cohort consisted of 294 patients. Transplant-related and outcome data were retrieved from the University of Minnesota Blood and Marrow Transplant Program Database, which prospectively collects these data on all patients transplanted at our institution. Additional data for this study were abstracted from patient medical records. Patients were treated on clinical protocols approved by our institutional review board.

Conditioning regimen intensity (myeloablative [MA] versus nonmyeloablative [NMA]) was prospectively determined by transplant protocols. Specific indications for HCT using NMA conditioning were older age (≥55 years for MRD and ≥45 years for UCB), presence of significant comorbidity (serious organ dysfunction, invasive mold infection within 3 months before transplantation or Karnofsky performance score of 50-60), or previous extensive prior therapy (>12 months of alkylator-based chemotherapy, >6 months of alkylator-based chemotherapy and extensive radiation, or history of autologous transplantation). Patients received UCB as a graft source if they had no HLA-compatible related donors. Our UCB selection criteria for adults have been previously published and allow the use of 2 UCB units to optimize cell dose, if necessary [12].

Patients were classified as having standard or high risk disease. Standard risk disease included acute leukemia in first complete remission (CR1), chronic myelogenous leukemia (CML) in first chronic phase, myelodysplastic syndrome (MDS) (refractory anemia only), and nonmalignant hematologic disorders; all other diagnoses were categorized as high-risk disease.

Conditioning Regimen and Supportive Care

MA and NMA regimens used at our institution have been described previously [13,14,16]. Briefly, patients undergoing MA MRD HCT received a regimen consisting of total-body irradiation (TBI) and cyclophosphamide (Cy), whereas recipients of MA UCB HCT received TBI, Cy, and fludarabine (Flu). NMA regimens for both MRD and UCB recipients consisted of TBI, Cy, and Flu. The TBI dose in MA regimens was 1320 cGy (165 cGy twice daily × 4 days) and in NMA regimens was 200 cGy (single fraction). Our GVHD prophylaxis and treatment regimens have also been described previously [17]. All patients received GVHD prophylaxis with cyclosporine (CaA; days −3 to at least +100), with trough levels maintained between 200 and 400 ng/mL and either methotrexate (MTX; in MA MRD recipients) or mycophenolate mofetil (MMF; in MA and NMA UCB and NMA MRD recipients; days −3 to at least +30).

Outpatient clinical evaluation to determine eligibility for transplantation was performed within 30 days prior to transplantation for all patients, and included history and physical examination, bone marrow biopsy and aspirate evaluation, assessment of organ function, determination of infectious markers, and appropriate radiologic imaging or other investigations for disease staging. Allogeneic HCT recipients were then admitted to the inpatient unit for initiating conditioning therapy and were discharged from the hospital after they had engrafted (absolute neutrophil count
[ANC] > 0.5 x 10^9/L for 3 days), had adequate oral intake, had transfusion or other infusion requirements that could be met as an outpatient, and had no complications requiring continued hospitalization. Frequency of outpatient follow-up was based on patient overall clinical condition and need for ongoing support (eg, transfusions, antibiotic infusions). All apheresis procedures for MRD peripheral blood stem cell (PBSC) collection were performed as an outpatient. All patients received antibacterial, antiviral, and antifungal prophylaxis and blood product and nutritional support per institutional guidelines. Granulocyte-colony stimulating factor (G-CSF) was administered to all patients until the ANC was >2.5 x 10^9/L for 2 days.

All patients are followed within our transplant program and institution from the time of pretransplant evaluation until at least 100 days posttransplant. Patients are required to stay within a 30-min driving distance from our transplant center, and accommodation is arranged for patients who do not live locally. All hospitalizations within the first 100 days are exclusively in a dedicated inpatient transplant unit that has resources for management of severe post-HCT complications (eg, mechanical ventilation, dialysis, pressor support). All outpatient visits within the first 100 days occur in our transplant clinic, which has infusion chairs and resources for performing minor procedures. Hence, our institutional accounting department captures all relevant medical costs for the first 100 days except costs for outpatient prescription drugs, including drugs administered through home-care services. Transplant-related care in this early posttransplant period was coordinated by our group of transplant physicians and midlevel providers who periodically rotate through both the inpatient and outpatient services and take care of all HCT recipients irrespective of their underlying diagnosis or transplant type. Therefore, individual provider practice variation did not have a major influence on costs within specific transplant types in our analysis.

Cost Data

Data regarding inpatient costs, days of hospitalization, and number of outpatient clinic visits were obtained from the institutional accounting department for all transplant-related costs prior to day 0 (from day -30) and until day 100 posttransplantation. Costs were determined by each hospital department's item and procedure specific costs and then summed from the itemized listing of each patient's hospital accounting record through day 100. Besides total cost of care (direct and indirect costs), specific categories of costs were also available. These categories included costs for "graft acquisition," "laboratory services," "radiologic investigations," "pharmacy services," "room and board," "blood components," and "other services." Examples of "other service" costs include costs for occupational therapy, physical therapy, and vascular access and operating room costs. We excluded costs for "physician services." Also, we could not account for outpatient prescription drug costs and did not include patient related nonmedical costs (eg, out-of-pocket costs, transportation, and accommodation) in our analysis.

Costs for "graft acquisition" consisted of costs for donor evaluation, apheresis procedure, and graft processing and storage for MRD HCT. For UCB recipients, this category included costs for searching the cord blood bank inventory, confirmatory HLA-typing of the cord blood unit, and shipping of the product. Median graft acquisition costs were $9566 for MRD and $68,830 for UCB transplantation. Although patients receiving matched unrelated donor HCT were excluded from this study, their median graft acquisition costs were $55,121. We excluded costs for graft acquisition from further cost analyses, as we wanted to specifically focus on the impact of posttransplant events on total costs. However, graft acquisition costs for a second graft infusion for graft failure or donor lymphocyte infusion for relapse within the first 100 days were included in cost analyses and were combined with the "other" category.

Because our data consisted of the actual dollar amount for cost incurred and given the relatively contemporary nature of our cohort, we did not adjust for inflation in our cost analyses.

Statistical Methods

The primary endpoint of this study was to compare medical costs among recipients of MA and NMA MRD and UCB transplantation. We also wanted to explore factors that were associated with increased costs of transplantation. To simplify comparison among different transplant categories, especially because of the variation in patient selection, risks for transplant-related complications and overall outcomes, costs are presented as cost per-day survived (in dollars).

Data are described as proportions or as median with range or interquartile range (lowest quartile-highest quartile). Comparison of patient, disease, and transplant characteristics was performed using chi-square, Fisher's exact, or Wilcoxon's rank sum test as appropriate. Cumulative incidence of engraftment, TRM, and GVHD was calculated by treating deaths from other causes as competing risks. The Kaplan-Meier method was used to plot curves for overall survival (OS). Multivariate Cox regression analysis was performed for OS after including the following variables: transplant type (main effect variable), age at HCT, sex, Karnofsky performance status at HCT, disease risk, previous HCT, cytomegalovirus (CMV)
status, HLA-match, acute GVHD (aGVHD) (grade iii-iv), graft failure, dialysis, mechanical ventilation, and hepatic veno-occlusive disease (VOD). Event times were measured from date of transplantation to date of death or last contact.

Analysis of variance (ANOVA) method was used to compare costs among different transplant types and was adjusted for the following variables: age at HCT, Karnofsky performance status at HCT, disease risk, previous HCT, CMV status, aGVHD (grade iii-iv), graft failure, dialysis, mechanical ventilation, hepatic VOD, duration of hospital stay (days of initial and any subsequent hospitalizations), and number of total medical encounters (days of hospitalization and outpatient clinic visits). HLA-match status correlated with transplant type and was not included as a separate variable. There were no significant interactions between transplant type and other predictor variables included in the ANOVA models.

All P-values reported are 2 sided. Analyses were performed using the SAS 9.1 software (Cary, NC).

RESULTS

Patient and Transplant Characteristics

Patient, disease, and transplant characteristics of our cohort are described in Table 1. As expected, recipients of NMA conditioning were older than those who received MA conditioning. The majority of patients who underwent UCB HCT (95%) received 2 cord blood units to optimize cell dose. MRD recipients were more likely to receive a 6/6 HLA matched graft (93% versus 9% for UCB). UCB recipients had slower neutrophil and platelet engraftment, had higher incidence of graft failure, and were more likely to have longer hospital stay compared to MRD recipients. The rates of major complications (dialysis, mechanical ventilation, or hepatic VOD) were similar among all types of allogeneic HCT.

Patient Survival and Outcomes

Probability of OS and cumulative incidences of TRM and aGVHD (grade iii-iv) in the first 100 days posttransplant were comparable among the 4 types of allogeneic HCT (Table 2). Transplant type was not predictive for OS on multivariate analysis (Table 3). Factors independently associated with increased risk of overall mortality included graft failure (relative risk [RR] 3.6, 95% confidence intervals [CI] 2.2-5.9), need for dialysis (RR 2.1, 95% CI 1.4-3.3), and need for mechanical ventilation (RR 4.4, 95% CI 3.1-6.2).

Costs of Transplantation

The median total cost of transplantation (excluding graft acquisition costs) within the first 100 days was $137,112 (interquartile range [IQR], 97,658-225,430) for MA and $84,824 (IQR, 52,247-151,906) for NMA allogeneic HCT, respectively (P < .001). The median total cost for UCB HCT was $137,564 (IQR, $81,486-$256,451) compared with $83,583 (IQR, $60,783-$123,581) for MRD HCT (P < .001). UCB HCT using either MA or NMA conditioning was associated with significantly higher costs than MRD HCT (Table 4). The median cost per day survived was $1,016 for MA and $612 for NMA MRD HCT and was $2082 for MA and $1156 for NMA UCB HCT (P < .001). For purposes of comparison, the median cost per day survived for matched unrelated donor HCT was $1586 for MA and $650 for NMA conditioning; however, these patients were excluded from further analyses because of small patient numbers.

The categories of cost for different transplant types are summarized in Figure 1. In general, the major contributors of cost for all transplant types were room and board and pharmacy services. MA and NMA UCB HCT recipients had longer hospitalizations, and as a result, had higher costs for room and board compared to recipients of MRD HCT. Pharmacy and laboratory services are more likely to be utilized during inpatient stay, and, hence, costs for these services were higher following UCB HCT. Because UCB transplant recipients also had a longer time to platelet engraftment, it was associated with higher blood component costs.

The contribution of various cost categories to total costs did not differ significantly among patients with low, intermediate, or high costs of care (Figure 2). However, the contribution of costs for blood components was relatively higher among patients whose care was the most expensive (total cost per day survived in the highest tertile, >$1805) compared with those with the least total costs (total cost per day survived in the lowest tertile, <$830).

Predictors of Cost

In multivariate analysis that adjusted for various factors that could influence costs (Table 5), MA UCB HCT was associated with higher costs than MA MRD HCT, but this difference was marginally significant (RR 1.3, 95% CI 1.1-1.5, P = .05). Interestingly, the costs of NMA MRD and NMA UCB HCT were similar to those of MA MRD HCT. More important predictors of costs were graft failure, need for dialysis, need for mechanical ventilation, and very long hospital stay. This is summarized in Table 6 and Figure 3, which highlights that patients with total costs in the highest tertile had a higher proportion of these risk factors compared to those with costs in the middle or lowest tertiles.

The median total cost per day survived for 23 patients with graft failure (MRD = 2, UCB = 21) was
Table 1. Patient, Disease, and Transplant Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>MA MRD</th>
<th>MA UCB</th>
<th>NMA MRD</th>
<th>NMA UCB</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>67</td>
<td>63</td>
<td>54</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>Median age, years</td>
<td>47</td>
<td>32</td>
<td>57</td>
<td>51</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Range</td>
<td>19-55</td>
<td>18-45</td>
<td>24-70</td>
<td>18-69</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 years</td>
<td>45 (67%)</td>
<td>63 (100%)</td>
<td>17 (32%)</td>
<td>51 (46%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>22 (33%)</td>
<td>0</td>
<td>37 (68%)</td>
<td>59 (54%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.86</td>
</tr>
<tr>
<td>Male</td>
<td>42 (63%)</td>
<td>35 (56%)</td>
<td>31 (57%)</td>
<td>64 (50%)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>25 (37%)</td>
<td>28 (44%)</td>
<td>23 (43%)</td>
<td>46 (42%)</td>
<td></td>
</tr>
<tr>
<td>KPS score at transplant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-100</td>
<td>58 (87%)</td>
<td>51 (81%)</td>
<td>37 (69%)</td>
<td>88 (60%)</td>
<td>.04</td>
</tr>
<tr>
<td>≤30</td>
<td>4 (6%)</td>
<td>4 (6%)</td>
<td>11 (20%)</td>
<td>16 (14%)</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td>5 (7%)</td>
<td>8 (13%)</td>
<td>6 (11%)</td>
<td>6 (6%)</td>
<td></td>
</tr>
<tr>
<td>Diagnoses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td>20 (30%)</td>
<td>28 (44%)</td>
<td>19 (35%)</td>
<td>33 (30%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>9 (13%)</td>
<td>18 (29%)</td>
<td>2 (4%)</td>
<td>6 (5%)</td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>13 (19%)</td>
<td>8 (13%)</td>
<td>14 (26%)</td>
<td>23 (19%)</td>
<td></td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
<td>2 (3%)</td>
<td>0</td>
<td>2 (4%)</td>
<td>13 (4%)</td>
<td></td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>1 (2%)</td>
<td>1 (2%)</td>
<td>3 (6%)</td>
<td>3 (3%)</td>
<td></td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>9 (13%)</td>
<td>1 (2%)</td>
<td>5 (9%)</td>
<td>16 (14%)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>13 (19%)</td>
<td>7 (13%)</td>
<td>9 (17%)</td>
<td>14 (13%)</td>
<td></td>
</tr>
<tr>
<td>Disease risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>37 (55%)</td>
<td>24 (38%)</td>
<td>15 (28%)</td>
<td>38 (33%)</td>
<td>.01</td>
</tr>
<tr>
<td>High</td>
<td>30 (45%)</td>
<td>39 (62%)</td>
<td>39 (72%)</td>
<td>72 (63%)</td>
<td></td>
</tr>
<tr>
<td>Previous transplant</td>
<td>0</td>
<td>0</td>
<td>15 (28%)</td>
<td>27 (24%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>CMV serological status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive (donor or recipient)</td>
<td>30 (45%)</td>
<td>14 (22%)</td>
<td>24 (44%)</td>
<td>11 (10%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Negative</td>
<td>37 (55%)</td>
<td>49 (78%)</td>
<td>30 (56%)</td>
<td>99 (90%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Graft source</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>63 (99%)</td>
<td>63 (100%)</td>
<td>51 (98%)</td>
<td>110 (100%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>4 (6%)</td>
<td>3 (6%)</td>
<td>9 (18%)</td>
<td>16 (14%)</td>
<td></td>
</tr>
<tr>
<td>UCB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single UCB</td>
<td>3</td>
<td>3</td>
<td>3 (9%)</td>
<td>3 (9%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Double UCB</td>
<td>60</td>
<td>60</td>
<td>60 (99%)</td>
<td>105 (99%)</td>
<td></td>
</tr>
<tr>
<td>HLA match*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/6</td>
<td>64 (95%)</td>
<td>6 (10%)</td>
<td>49 (91%)</td>
<td>9 (8%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>5/6</td>
<td>3 (5%)</td>
<td>17 (27%)</td>
<td>9 (9%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>6/6</td>
<td>0</td>
<td>40 (64%)</td>
<td>0</td>
<td>70 (64%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median time to ANC engraftment, days</td>
<td>23 (48%)</td>
<td>23 (48%)</td>
<td>3 (6%)</td>
<td>3 (6%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Range</td>
<td>9-25</td>
<td>7-38</td>
<td>0-30</td>
<td>0-60</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median time to platelet engraftment, days</td>
<td>56 (56%)</td>
<td>56 (56%)</td>
<td>15 (15%)</td>
<td>47 (47%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Range</td>
<td>13-70</td>
<td>33-100</td>
<td>0-100</td>
<td>0-100</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Graft failure</td>
<td>2 (3%)</td>
<td>12 (19%)</td>
<td>0</td>
<td>9 (5%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Second graft infusion</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Medical encounters in first 100 days</td>
<td>39 (39%)</td>
<td>48 (48%)</td>
<td>23 (23%)</td>
<td>38 (38%)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median hospital stay, days</td>
<td>30-47</td>
<td>40-76</td>
<td>18-37</td>
<td>24-60</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median clinic visits, days</td>
<td>30</td>
<td>19</td>
<td>28</td>
<td>28</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>17-38</td>
<td>9-32</td>
<td>13-38</td>
<td>9-38</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Median total encounters, days</td>
<td>73</td>
<td>75</td>
<td>54</td>
<td>70</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>53-83</td>
<td>59-93</td>
<td>42-67</td>
<td>57-91</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>Major complications</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialysis</td>
<td>9 (13%)</td>
<td>10 (16%)</td>
<td>4 (7%)</td>
<td>10 (9%)</td>
<td>.39</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>20 (30%)</td>
<td>25 (40%)</td>
<td>13 (24%)</td>
<td>28 (26%)</td>
<td>.19</td>
</tr>
<tr>
<td>Hepatic veno-occlusive disease</td>
<td>5 (8%)</td>
<td>1 (2%)</td>
<td>9 (3%)</td>
<td>2 (3%)</td>
<td>.09</td>
</tr>
<tr>
<td>Median follow-up, months</td>
<td>31</td>
<td>25</td>
<td>38</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>12-53</td>
<td>12-50</td>
<td>12-53</td>
<td>12-49</td>
<td></td>
</tr>
</tbody>
</table>

MRD indicates matched related donor; UCB, umbilical cord blood; MA, myeloablative; NMA, nonmyeloablative; KPS, Karnofsky performance status; CMV, cytomegalovirus; HLA, human leukocyte antigen; ANC, absolute neutrophil count.

*Worst match for recipients of double UCB transplant.

$6976 (IQR, 5074-8698). In comparison, the median cost per day survived for patients who did not experience graft failure was $1105 (IQR, 679-2149) (P < .001). UCB HCT was associated with higher total costs even after patients with graft failure were excluded; median cost per day survived for MA and NMA MRD HCT recipients was $1005 and $612, whereas that for UCB HCT recipients was $1703 and $1115, respectively (P < .001).

Patients who received dialysis had a median total cost per day survived of $4764 (IQR, 1194-6976) compared to $1102 (IQR, 678-2209) among those who
did not receive dialysis (P < .001). Similarly, patients who received and did not receive mechanical ventilation had total cost per day survived of $5099 (IQR, 1287-7570) and $977 (IQR, 614-1508), respectively (P < .001).

**DISCUSSION**

In our contemporary cohort of adult HCT recipients, we observed the absolute costs of MA and NMA UCB transplantation to be higher than MA and NMA MRD transplantation. However, the costs of transplantation were primarily driven by severe posttransplant complications (graft failure, dialysis, and mechanical ventilation) and prolonged inpatient stay. UCB recipients have longer time to neutrophil and platelet engraftment than MRD recipients. Because 1 of the main endpoints for hospital discharge is engraftment, it is not surprising that UCB recipients had longer inpatient stay with its associated costs (room and board, pharmacy services, laboratory services, and blood components). Also, graft failure was more common following UCB HCT. Graft failure does increase the duration of hospitalization, and prolonged pancytopenia can increase the risk of infectious complications and transfusion requirements with a resultant increase in costs. The cost of a second graft infusion, especially UCB, also adds to this expense. Because the rates of severe complications (excluding graft failure) were similar among the 4 groups, the cost differences between MRD and UCB are more likely a result of prolonged hospitalization because of delayed engraftment and graft failure rather than complications. Our study did not address long-term costs of UCB transplantation. There is emerging data that UCB HCT is associated with a lower risk of chronic GVHD (cGVHD) [14,17]. Whether this would translate to lower or comparable costs versus MRD or matched unrelated donor HCT over an extended period of time needs to be investigated.

The use of less intense NMA conditioning does not necessarily translate to lower costs. In our study, the cumulative incidence of TRM was similar between all allogeneic transplant types, regardless of donor source or conditioning regimen intensity. Also, there was no difference in the rates of severe aGVHD, dialysis, mechanical ventilation, or hepatic VOD. Therefore, the older age and/or poor health status of NMA HCT recipients may offset the lesser toxicity of a reduced-intensity conditioning (RIC) regimen. We observed similar costs for MA and NMA MRD HCT. The occurrence of severe complications was a more important

---

**Table 2. Univariate Analysis for Posttransplant Outcomes**

<table>
<thead>
<tr>
<th></th>
<th>MA MRD</th>
<th>MA UCB</th>
<th>NMA MRD</th>
<th>NMA UCB</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall survival</td>
<td>81 (71%-91%)</td>
<td>79 (59%-85%)</td>
<td>78 (67%-89%)</td>
<td>78 (70%-86%)</td>
<td>.95</td>
</tr>
<tr>
<td>Treatment-related mortality</td>
<td>21 (1%-31%)</td>
<td>29 (17%-40%)</td>
<td>20 (10%-31%)</td>
<td>19 (12%-27%)</td>
<td>.55</td>
</tr>
<tr>
<td>Grade II-IV acute GVHD*</td>
<td>15 (6%-24%)</td>
<td>24 (13%-34%)</td>
<td>22 (11%-33%)</td>
<td>17 (10%-24%)</td>
<td>.45</td>
</tr>
</tbody>
</table>

MRD indicates matched related donor; UCB, umbilical cord blood; MA, myeloablative; NMA, nonmyeloablative; GVHD, graft-versus-host disease. *Cumulative incidence estimate.

---

**Table 3. Multivariate Analysis for Overall Survival at 100 Days**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Relative Risk (95% Confidence Interval)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA MRD</td>
<td>1.0</td>
<td>.59</td>
</tr>
<tr>
<td>MA UCB</td>
<td>1.1 (0.74-1.6)</td>
<td>.85</td>
</tr>
<tr>
<td>NMA MRD</td>
<td>0.8 (0.5-1.2)</td>
<td>.21</td>
</tr>
<tr>
<td>NMA UCB</td>
<td>1.0 (0.6-1.5)</td>
<td>.98</td>
</tr>
<tr>
<td>Graft failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Yes</td>
<td>3.6 (2.2-5.9)</td>
<td>.001</td>
</tr>
<tr>
<td>Dialysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2.1 (1.4-3.3)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4.4 (3.1-6.2)</td>
<td></td>
</tr>
</tbody>
</table>

MRD, matched-related donor; UCB, umbilical cord blood; MA, myeloablative; NMA, nonmyeloablative. *Other variables considered in the model included age at transplantation, sex, KPS score at transplantation, disease risk, history of previous transplant, CMV status, HLA match, graft source, acute graft-versus-host disease, and occurrence of hepatic veno-occlusive disease.

---

**Table 4. Costs of Allogeneic Hematopoietic Cell Transplantation**

<table>
<thead>
<tr>
<th>Transplant Type*</th>
<th>N</th>
<th>Median</th>
<th>Interquartile Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloablative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRD</td>
<td>67</td>
<td>1016</td>
<td>796-2232</td>
</tr>
<tr>
<td>UCB</td>
<td>63</td>
<td>2082</td>
<td>1306-6219</td>
</tr>
<tr>
<td>MUD</td>
<td>7</td>
<td>1596</td>
<td>1283-3892</td>
</tr>
<tr>
<td>Nonmyeloablative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRD</td>
<td>54</td>
<td>612</td>
<td>473-1023</td>
</tr>
<tr>
<td>UCB</td>
<td>110</td>
<td>1156</td>
<td>616-2472</td>
</tr>
<tr>
<td>MUD</td>
<td>5</td>
<td>650</td>
<td>618-763</td>
</tr>
</tbody>
</table>

MRD indicates matched related donor; UCB, umbilical cord blood; MUD, matched unrelated donor. *Excluding costs of graft acquisition. **Recipients of matched unrelated donor grafts were excluded from further analyses.
driver of costs than conditioning regimen intensity. Other investigators have recently conducted cost analyses comparing MA and NMA HCT. Saito et al. [8] included 90 NMA and 185 MA HCT recipients transplanted between 2000 and 2003 in their retrospective analysis. They showed that NMA HCT costs approximately $33,030 less, and was associated with 16 fewer days of hospitalization than myeloablative HCT within the first year after transplantation. In another study, Cordonnier et al. [2] evaluated the 1-year costs of transplantation in 11 NMA and 12 MA HCT recipients with acute myelogenous leukemia (AML) who were enrolled on a prospective trial between 1998 and 2003. There was a trend toward lower costs of transplantation in the first 6 months for NMA conditioning, but the costs from 6-12 months were higher because of late complications and readmissions and there was no difference in the costs of NMA and
Table 5. Multivariate Analysis for Predictors of Costs of Allogeneic Transplantation

<table>
<thead>
<tr>
<th>Variable*†</th>
<th>Relative Risk (95% Confidence Intervals)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MA MRD</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>MA UCB</td>
<td>1.3 (1.1-1.5)</td>
<td>.05</td>
</tr>
<tr>
<td>NMA MRD</td>
<td>1.0 (0.9-1.2)</td>
<td>.02</td>
</tr>
<tr>
<td>NMA UCB</td>
<td>1.0 (0.8-1.2)</td>
<td>.06</td>
</tr>
<tr>
<td>Graft failure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.8 (1.7-1.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Diastasis</td>
<td>No</td>
<td>1.0</td>
</tr>
<tr>
<td>Yes</td>
<td>1.3 (1.1-1.5)</td>
<td>.05</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1.3 (1.2-1.4)</td>
<td>.004</td>
</tr>
<tr>
<td>Hospital stay, tertiles‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;32 days</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>32-48 days</td>
<td>1.0 (0.8-1.2)</td>
<td>.06</td>
</tr>
<tr>
<td>&gt;48 days</td>
<td>2.1 (1.9-2.3)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

MRD indicates matched related donor; UCB, umbilical cord blood; MA, myeloablative; NMA, nonmyeloablative.
*Other variables considered in the model included age at transplantation, KPS score, transplantation, disease risk, history of previous transplant, CMV status, acute graft-versus-host disease, hepatic veno-occlusive disease, and total medical encounters in days (by tertiles). Graft source and HLA match correlated with transplant type and were not included in the models as separate variables.
†Excluding costs of graft acquisition.
‡Total hospital stay in first 100 days posttransplantation.

MA HCT at 1 year posttransplantation. Differences in patient population and transplant techniques (eg, conditioning regimens) could explain the discrepant results from our and these 2 published studies. Some observational studies have shown equivalent long-term survival among patients receiving MA and NMA HCT for selected diseases [16,18,19]. With similar outcomes, the transplant modality with lesser costs and lesser morbidity would be preferred. Hence, more studies to better understand cost differences between MA and NMA conditioning regimens are needed and any randomized trials comparing these 2 modalities should include economic and quality of life endpoints.

The role of complications in increasing costs of transplantation has been described previously. In a study of 315 MA allogeneic HCT recipients transplanted between 2000 and 2004, [7], showed that the mean cost of transplantation in their cohort was $779,222, but severe complications increased total costs by an average of $20,228. [5], have also shown that complications are associated with higher costs. Their study included 181 patients who received a n MA allogeneic HCT between 1994 and 1997; the median initial inpatient cost was $105,300 and occurrence of infection, hepatic VOD, aGVHD, and death were predicted to add between $15,300 and $28,100 each to the costs of transplantation. Prevention and early recognition and management of complications, where possible, can decrease the costs of transplantation. Esperou et al. [3], in a study of 85 MA allogeneic HCT recipients from 1998-2000, have also shown that predictors of higher costs (adding an average $20,000/patient) include transplant related complications, GVHD, and repeated infections.

Several limitations have to be considered in the interpretation of our analysis. There exists considerable practice variation in HCT among transplant centers, and our results may not be generalizable. Also, we captured costs within the first 100 days following transplantation and did not consider costs of long-term care or management of cGVHD and its complications. Other studies have shown that the costs of transplantation are largely concentrated within the first 100 days [7].

We selected the early posttransplant period for investigation because all medical care is conducted exclusively at our center. Nevertheless, we could not account for costs of outpatient prescription drugs and home-care services. Transplant conditioning and GVHD prophylaxis and management regimens were dictated by specific protocols, and supportive care was based on established guidelines, limiting the impact of practice variation on costs.

We excluded costs of graft acquisition in cost analyses because the characteristics of graft procurement, storage, and processing are very different for MRD and UCB. Given the resources needed for UCB collection and storage, UCB graft acquisition is much more expensive than MRD. The contribution of graft
acquisition costs to total costs cannot be ignored, especially because of its large dollar amount, and any strategies to increase the cost effectiveness of UCB HCT will also have to address these costs.

Because of the relatively small number of patients who received a matched unrelated donor HCT, we could not perform detailed analyses comparing costs between matched unrelated donor and UCB transplantation. Because UCB is primarily considered among various alternative donor options for patients without an MRD, future cost analyses comparing matched unrelated donor and UCB HCT will be important. In our unadjusted descriptive analysis, transplantation with matched unrelated donor was more expensive than MRD but less expensive than UCB for both MA and NMA conditioning regimens.

In conclusion, allogeneic HCT is a costly procedure. In the first 100 days after transplantation, the costs of MA and NMA MRD and nonmyeloablative UCB transplantation are similar, whereas MA UCB HCT is more expensive. Severe complications, graft failure, and prolonged hospitalization are the major contributors to total costs in the early posttransplant period. Increased costs of UCB HCT are primarily because of longer hospitalization for delayed engraftment and graft failure. Strategies to decrease the risk of severe complications would reduce the overall costs of transplantation in general. Methods to enhance engraftment and decrease the risk of graft failure in recipients of UCB HCT would make this procedure more cost effective.

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REFERENCES


Unrelated donor umbilical cord blood transplantation for the treatment of hematologic malignancies
Craig Sauter and Juliet N. Barker

Introduction
Allogeneic hematopoietic stem cell transplantation (HSCT) is indicated for the treatment of many high-risk hematologic malignancies. Unfortunately, application of this treatment is limited by a lack of suitable donors. Only 25% of patients have a HLA-matched sibling donor suitable for HSC donation and despite the many millions of volunteer donors registered in the unrelated donor (URD) pool, many patients do not have an adequately human leukocyte antigen (HLA)-matched URD using high-resolution donor–recipient HLA matching especially patients from racial and ethnic minorities [1]. Additionally, the procurement of an URD graft can often take months, whereas patients with hematologic malignancies frequently require urgent transplantation.

Umbilical cord blood (UCB) can reconstitute hematopoietic stem cells in adults following both myeloablative [2–8] and reduced-intensity/nonmyeloablative (NMA) [9,10,11] conditioning and has the advantage of ready availability. Importantly, in marked contrast to the transplantation of URD HSC [12], the reduced stringency of the required HLA match with UCBT translates to the potential to extend allogeneic HSCT access to patients without other suitable donors (Table 1). This factor alone, along with multiple other attributes, frequently outweighs the disadvantages of UCBT as compared with URD HSCT (summarized in Table 2), thus accounting for the rapid expansion of use of this relatively new HSC source (Fig. 1). This review will outline the current status of UCBT as compared with URD HSC transplantation as well as discussing current issues associated with the transplantation of this HSC source.

Outcome of umbilical cord blood transplantation compared with unrelated donor bone marrow transplantation
Although no randomized controlled trials have compared UCBT and URD transplantation, retrospective studies have compared single-unit UCBT with URD bone marrow transplantation (BMT) using myeloablative conditioning in adults and children. In 2004, Laughlin et al. [5] and Rocha et al. [6] reported the first comparisons between UCBT and URD transplantation in adults. The
Table 1 Ancestry of 48 umbilical cord blood transplantation recipients at Memorial Sloan-Kettering Cancer Center October 2005–April 2008

<table>
<thead>
<tr>
<th>Ancestry</th>
<th>UCBT recipients, n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northwest Europe</td>
<td>4</td>
</tr>
<tr>
<td>Eastern Europe</td>
<td>6</td>
</tr>
<tr>
<td>Southern Europe</td>
<td>7</td>
</tr>
<tr>
<td>European Mz</td>
<td>7</td>
</tr>
<tr>
<td>Asian</td>
<td>9</td>
</tr>
<tr>
<td>African</td>
<td>6</td>
</tr>
<tr>
<td>Middle Eastern</td>
<td>1</td>
</tr>
<tr>
<td>Hispanic/Latino</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
</tr>
</tbody>
</table>

Patients were offered UCBT if allogeneic transplant was indicated and no suitably HLA-matched-related or unrelated volunteer donor was available. Notably, 60% of patients were of non-North Western European ancestry with 50% of patients being non-European. In addition, the four patients of North-Western European ancestry had proven or potential 9/10 or 10/10 HLA-A, B, C, DRB1, DQ allele-matched-unrelated volunteer donors but received UCB due to transplant urgency (n = 1) or patient preference (n = 3). UCBT grafts were 4/6 HLA-matched at A and B antigens and DRB1 alleles. HLA, human leucocyte antigen; UCBT, umbilical cord blood transplantation.

American series found comparable survival after UCBT (n = 150) and one antigen-mismatched BMT (n = 83) [5], whereas the European reported that HLA-mismatched adult UCBT (n = 98) was associated with comparable survival to 6/6 HLA antigen-matched BMT (n = 584) [6]. In contrast, a Japanese series reported by Takahashi et al. [7] demonstrated superior transplant-related mortality (TRM) and disease-free survival (DFS) in 68 adult UCBT as compared with 45 URD BMT recipients.

More recently, Eapen et al. [13**] have analyzed the outcomes of 503 UCBT recipients of 4/6 HLA-A, B antigen and DRB1 allele-matched single-unit UCBT as compared with those of URD BMT in children below 16 years of age with leukemia. Most notably, in a subset analysis comparing UCBT outcomes with the 116 recipients of the ‘gold standard’ of 8/8 HLA allele-matched bone marrow, the 35/6/6 HLA-matched UCBT recipients had significantly higher 5-year DFS, with 201/5/6 and 267 4/6 UCBT having comparable DFS with that of 8/8 allele-matched BMT recipients and demonstrated a robust protection against relapse (Table 3).

These findings support UCBT as an alternative to URD BMT in children. Further, if engraftment after UCBT is improved, it suggests that pediatric UCBT may be a superior HSC for the treatment of leukemia. In adults, the American and European comparisons, although establishing UCBT as a potential alternative to URD BMT, have highlighted that the poor engraftment and high TRM must be addressed for this HSC to be widely adopted. At the current time, whether UCBT will be offered to a patient will be frequently determined by the relative availability of a closely HLA-matched (7–8/8 alleles) URD versus an UCBT graft of at least 4/6 HLA-A, B antigen and DRB1 allele match and adequate dose; and the experience and research bias of the transplant center.

Table 2 Relative advantages and limitations of unrelated umbilical cord blood as a hematopoietic stem cell source as compared with transplantation with unrelated volunteer donors

<table>
<thead>
<tr>
<th>Advantage of UCB</th>
<th>Comparison with URD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid access without the problem of donor availability (admirably reduces around patient)</td>
<td>Major advantage over URD</td>
</tr>
<tr>
<td>Ability to reschedule easily</td>
<td>Advantage over URD</td>
</tr>
<tr>
<td>Reduced requirement for HLA match at high resolution</td>
<td>Major advantage over URD</td>
</tr>
<tr>
<td>Less severe GVHD with chronic GVHD easier to treat</td>
<td>Similar to URD</td>
</tr>
<tr>
<td>Preserved graft-versus-leukemia effect</td>
<td>Major advantage over URD</td>
</tr>
<tr>
<td>Potential to build inventory from all racial groups</td>
<td>Comparison with URD</td>
</tr>
<tr>
<td>Limitation of UCB</td>
<td>Major disadvantage over URD</td>
</tr>
<tr>
<td>Limited cell dose</td>
<td>UCB transplantation also has limited availability to minorities</td>
</tr>
<tr>
<td>Limited inventory to enable at least 4/6 HLA matches of adequate dose for patients of all races</td>
<td>Disadvantage over URD</td>
</tr>
<tr>
<td>Potential for variable unit quality at thaw</td>
<td>Disadvantage over UBD for cellular therapies</td>
</tr>
<tr>
<td>and naïve immune system</td>
<td></td>
</tr>
</tbody>
</table>

GVHD, graft-versus-host disease; HLA, human leucocyte antigen; UCB, umbilical cord blood; URD, unrelated donor.

*Not yet examined for acute GVHD.
needed for adult UCBT recipients this approach is equally as relevant to many children given graft failure is still a devastating feature of many pediatric UCBT series and many larger children will only have access to units of relatively low cell doses that similarly challenge adult UCBT recipients.

Initial investigation with double-unit UCBT following a total-body irradiation (TBI)-based myeloablative conditioning regimen yielded a DFS of 57% [95% confidence interval (CI) 35–79] in 23 leukemia patients (median age 24 years), with a DFS of 72% if transplanted in remission [19]. Updated survival data after myeloablative double-unit UCBT in high-risk hematologic malignancies is shown in Fig. 2. Interestingly, both engraftment and survival was improved after double-unit UCBT as compared with historical single-unit controls [4] despite only one of two relatively low cell dose units being responsible for sustained donor engraftment in the vast majority of patients. This raises the possibility that the ‘losing’ unit is somehow facilitating the engraftment of the engrafting or ‘winning’ unit. However, it is important to note that the single-unit historical controls were transplanted using cyclophosphamide and TBI with antithymocyte globulin (ATG) and cyclosporine-A (CSA)/methylprednisolone as immunosuppression. In contrast, though the double-unit transplants were also performed with cyclophosphamide/TBI and CSA, the ATG and MP were substituted with low-dose fludarabine (Flu) and mycophenolate mofetil (MMF). This raises the possibility that some of the advantage of double-unit UCBT was due to changes in the preparative regimen and immune suppression independent of the graft. This question is therefore being investigated in the Bone Marrow Transplant Clinical Trials Network (BMT CTN) single versus double-unit randomized trial in children in the United States utilizing the cyclophosphamide/Flu/TBI and CSA/MMF regimen. Importantly, however, this study may not fully answer the question of the utility of double-unit UCBT in adults. A major question for the field currently is how double-unit UCBT compares with URD peripheral blood stem cell (PBSC) transplantation in adult patients and should be studied in the near future.

Table 3: Comparison of outcomes after 8/8 HLA allele-matched unrelated donor bone marrow transplantation and 4–6 A, B antigen, DRB1 allele-matched umbilical cord blood transplantation in children with acute leukemia [13**]

<table>
<thead>
<tr>
<th>HSC source</th>
<th>TRM (%)</th>
<th>Relapse (%)</th>
<th>DFS (%)</th>
<th>Overall survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/8-matched bone marrow (n = 116)</td>
<td>19</td>
<td>41</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>UCB (n = 503)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6/6</td>
<td>6</td>
<td>34</td>
<td>60</td>
<td>63</td>
</tr>
<tr>
<td>5/6 × 10^7 NCD/kg</td>
<td>29</td>
<td>31</td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td>4/6 &lt;3.0 × 10^7 NCD/kg</td>
<td>43</td>
<td>21</td>
<td>37</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>20</td>
<td>33</td>
<td>33</td>
</tr>
</tbody>
</table>

Survival data are reported at 5 years after transplant. DFS, disease-free survival; HSC, hematopoietic stem cell; TRM, transplant-related mortality; UCB, umbilical cord blood.
Figure 2 Survival after myeloablative double-unit umbilical cord blood transplantation (n = 83)

Patients were conditioned with cyclophosphamide 120 mg/kg, TBI 1320 cGy, fludarabine 75 mg/m² with CSA/MMF, CI confidence interval; CSF, cyclosporine-A; DFS, disease-free survival; MMF, mycophenolate mofetil; TBI, total-body irradiation. University of Minnesota data (reproduced with permission from Professor John Wagner, University of Minnesota, 2007).

The double-unit UCBT experience has raised unique questions about transplant biology especially given that preliminary University of Minnesota data have suggested that this strategy has also been associated with a reduced risk of relapse as compared with single-unit UCBT [20]. To date, no reliable factor has been able to predict which unit will predominate in engraftment after double-unit transplantation. Interestingly, Scaradavou et al. [21] have recently demonstrated an association between UCB unit CD34+ cell viability after thaw [as measured by flow cytometric 7-amino-actinomycin D (7-AAD) staining] and unit predominance. In this analysis of 26 double-unit UCBTs, although the factor determining unit predominance when both units of a double-unit graft have high viability was unclear, units with low viability did not engraft (P = 0.007). Such data suggest that the reason that double-unit UCBT is efficacious may simply be because it increases the chance that the patient will receive at least one unit of high viability and thus with engraftment potential. This introduces the concept that postthaw CD34+ cell viability could be an effective measure of unit quality and has the advantage that, unlike colony-forming assays, is available on the day of transplant. Postthaw unit quality is a relatively new variable to be considered in the field of UCBT and will be a critical area of investigation for the future. If these findings are confirmed it would suggest that double-unit UCBT may be indicated even in children given the viability of a unit that appears satisfactory from the standpoint of HLA-matched and TNC dose cannot be predicted prior to thaw.

Although the poor engraftment and high TRM associated with low-infused TNC dose in single-unit UCBT has understandably led to a focus on strategies to augment graft cell dose, unit selection is complicated by the fact that both engraftment and TRM are also influenced by HLA match. For example, in an analysis of 989 single-unit myeloablative UCBT recipients facilitated by the National Cord Blood Program of the New York Blood Center (NYBC), HLA-A, B antigen and DRB1 allele match was associated with significantly improved engraftment, a lower incidence of severe acute graft-versus-host disease (GVHD), lower TRM, and improved DFS [22]. Eurocord analyses have also found that HLA match is associated with significantly improved engraftment and lower TRM [8].

These findings lead to the question of how to 'trade-off' HLA match with TNC dose when selecting UCB units for transplantation. Although this issue is yet to be fully resolved it is intriguing that in the NYBC analysis referenced above [22] 6/6 HLA-matched UCBT recipients (any dose) had superior DFS to recipients of either 5/6 units at least 2.5 x 10⁷ TNC/kg or 4/6 units at least 5 x 10⁶ TNC/kg. Further, recipients of 5/6 at least 2.5 x 10⁷ TNC/kg units had a comparable DFS to those of 4/6 HLA-matched units with a TNC at least 5.0 x 10⁷/kg [although with less severe acute GVHD (aGVHD)]. This raises the concept of a 'sliding scale' in unit selection with HLA-matched compensating for lesser cell dose (or conversely that the greater the HLA mismatch the greater the cell dose required), and prompts a unit selection algorithm of 6/6 units followed by 5/6 units above 2.5 x 10⁷/kg, and 4/6 units above 5.0 x 10⁶/kg. However, many patients will not have access to such units, and some patients with such optimal units will still not engraft. One strategy to address this limitation that is being investigated in a Center for Bone Marrow Transplant Research (CIBMTR) sponsored study is to prioritize HLA match above a cell dose threshold of 1.5 x 10⁷/kg but to augment engraftment by the infusion of two units.

Graft-versus-host disease after umbilical cord blood transplantation

Although GVHD remains one of the leading causes of TRM in allogeneic HSCT, UCBT has consistently demonstrated a lower than expected incidence of acute and chronic GVHD (cGVHD) [2–8] especially given the considerable degree of HLA mismatch if high-resolution
typing is considered [23]. In comparison with URD BMT, Exepen et al. [13*] reported a similar incidence of grade 2–4 acute and chronic GVHD in pediatric 8/8 allele-matched BMT and 4–6/6 A, B antigen, DRB1-matched UCBT. In adult recipients, Laughlin et al. [5] found a similar incidence of grade 2–4 aGVHD and a lesser incidence of extensive cGVHD as compared with HLA-matched BMT recipients. In contrast, Rocha et al. [6] reported a lower risk of grade 2–4 aGVHD in adult UCBT as compared with HLA-matched BMT recipients with a relative risk of cGVHD of 0.64 after URD BMT although this did not reach significance (P = 0.11). Takahashi et al. [7] have reported similar findings to the Rocha et al. study [6], and more recently these investigators have even reported a significantly lower incidence of grade 3–4 aGVHD and extensive cGVHD after predominantly HLA-matched-related donor HSC transplantation and mismatched URD donor UCBT in adults [24*].

Of further interest beyond the incidence of GVHD is the nature of this disease after UCBT and its response to therapy. Although this has not yet been examined for aGVHD, Arora et al. [25*] found more frequent responses of cGVHD to therapy in 47 UCBT as compared with predominantly HLA-matched URD BMT recipients at 2 months (74 versus 48%, P = 0.005), 6 months (78 versus 49%, P = 0.001) and 1 year (72 versus 51%, P = 0.03) following cGVHD diagnosis. UCBT cGVHD was also associated with a lower TRM (11 versus 27% with URD BMT). It is likely that the findings in this study in favor of UCBT may have even been more pronounced if the URD transplant recipients had received PBSC as the HSC source rather than bone marrow, and given the wide adoption of PBSC (Fig. 1) comparisons of both aGVHD and cGVHD after UCBT to recipients of URD PBSC should be a priority for the future.

The exact reasons for the relatively low incidence of GVHD after UCBT are unknown but likely result from the functional immaturity of the infused lymphocytes including decreased cytotoxicity, an altered cytokine profile, decreased HLA expression and increased regulatory T cells. Of even more interest is to understand the biology of why UCBT is associated with a retained graft-versus-leukemia effect despite the GVHD reduction.

Infectious complications after umbilical cord blood transplantation and immune recovery
Opportunistic infections are a significant cause of TRM in HSCT regardless of graft source. However, studies have revealed varying results in the assessment of infection risk after UCBT as compared with other HSC sources. A University of Minnesota analysis revealed equal incidences of one or more serious infections in unmodified bone marrow [81% (95% CI 65–97%), T-cell depleted [83% (95% CI 60–100%)] and UCB [90% (95% CI 74–100%)] pediatric transplant recipients (P = 0.48) in the first 2 years after transplant, with no significant differences overall when taking all serious infections into account [26]. Further, more recently this group has reported a similar risk of cytomegalovirus (CMV) infection in recipients of UCB, bone marrow and PBSC grafts [27]. Another study [28] has reported increased incidences of severe infection in 48 adult UCBT (85% risk) as compared with 144 adults URD HSC transplant (69% risk) recipients, although day 100 and 3-year infection-related mortality did not differ between HSC sources.

Regardless of the specifics of such comparisons infection is a major challenge in UCBT and at many centers infection-related mortality is now the most frequent cause of death in UCBT with the majority of deaths occurring within the first 3–4 months [29*]. Although improved engraftment with new preparative regimens and double-unit grafts, and aggressive supportive care to abrogate neutropenic sepsis and prevent fungal infections by the use of extended spectrum azoles have led to decreased infection-related TRM, viral infections remain a critical challenge in the early posttransplantation period. For example, Duke University analyzed 330 pediatric patients undergoing UCBT and reported most deaths within the first 6 months after transplant being attributable to opportunistic infection, of which more than half were secondary to CMV or adenoovirus [29*].

Important in interpreting the infectious complications and immune recovery seen after UCBT is not only considering patient and unit characteristics but also what preparative regimen and immune suppression was used. The use of ATG [30,31], corticosteroids, or both for GVHD prophylaxis, for example, appears to be associated with impaired immune recovery and increased risk of severe infection. How to augment immune reconstitution is a major question in the field of UCBT today and assume even greater importance with the recognition that improved immune recovery has also been associated with protection against leukemic relapse [32]. Cellular therapy approaches, although clearly challenging given the naïve neonatal immune system, may yet show promise in the future. In the interim, improved preparative regimen/immune suppression and aggressive supportive care including surveillance for viral reactivation is mandatory in the care of UCBT patients in the early posttransplant period.

Reduced-intensity or nonmyeloablative conditioning
Reduced-intensity conditioning (RIC) or NMA HSCT has been investigated as a method to offer the potential benefit
of a graft-versus-malignancy effect to older, more heavily pretreated, more infirm patients, or all with less toxicity. Early series from the University of Minnesota demonstrated that UCBT after NMA conditioning was feasible [9]. However, it was observed that there was a strong association between recent exposure to combination chemotherapy or a prior autologous transplant and the likelihood of sustained donor engraftment (Fig. 3) [33]. This group has recently updated their NMA UCBT experience [10*]. One-hundred and ten patients (median age 51 years) with high-risk or advanced leukemias, myelodysplasia and Hodgkin's or non-Hodgkin's lymphoma unsuitable for myeloablative conditioning received cyclophosphamide 50 mg/kg, Flu 200 mg/m², and TBI 200 cGy with immune suppression of CSA/MMF. Eighty-five percentage of patients received double-unit grafts to attain a target TNC dose of at least 3.0 × 10⁶/kg. In this high-risk patient group, TRM was 26% (95% CI 18–34) at 3 years with an

Figure 3 Association between prior chemotherapy exposure and sustained donor engraftment after nonmyeloablative umbilical cord blood transplantation

CI, confidence interval; UCBT, umbilical cord blood transplantation. University of Minnesota data.

Figure 4 A schema representing the relationships between current umbilical cord blood banks

BMDW, bone marrow donors worldwide; NMDP, National Marrow Donor Program. Reproduced with permission from Mary Halet, NMDP, April 2008.
overall survival of 45% (95% CI 34–56) and progression-free survival (PFS) of 38% (95% CI 28–48) at 3 years. Interestingly, the PFS was significantly higher in recipients of double-unit [39% (95% CI 27–51)] as compared with single-unit [24% (95% CI 4–44)] grafts. Further to these findings, the Minnesota group has also reported comparable PFS after RIC allograft in recipients older than 55 years of matched-related donor (n = 47) or UCB (n = 43) grafts with 3-year PFS of 30% (95% CI 16–44) and 34% (95% CI 19–48), respectively [34].

Ballen et al. [11] have also investigated double-unit UCBT utilizing a RIC regimen of Flu 180 mg/m² with melphalan 100 mg/m², rabbit ATG, and CSA/MMF in advanced hematologic malignancies or severe aplastic anemia reporting a 100-day TRM of 14% and a promising 1-year DFS of 67% in 21 patients. The outcomes from these series appear comparable to previously published series of RIC/NMA transplantation using volunteer donors but this will need to be studied formerly in randomized studies in the future. For the meantime, major questions in the field of RIC/NMA UCBT are: how to ensure engraftment in patients (such as those with myelodysplasia, myelofibrosis and acute myelogenous leukemia who have received a single induction) without intensive prechemotherapy (especially given the addition of ATG to the NMA preparative regimen) as a strategy to augment engraftment is associated with a high incidence of Epstein-Barr virus posttransplant lymphoproliferative disease [30]; and what is the efficacy of RIC/NMA UCBT in specific disease entities.

Umbilical cord blood banking
As a counterpart to the increased adoption of UCB as an alternative HSC source, the number of units banked worldwide continues to increase with at least 250,000 units for unrelated recipient use banked to date. However, the UCB search continues to be a challenge with no centralized search mechanism to access all units in the global inventory (Fig. 4) and no international regulation to ensure uniform standards from bank to bank. Notably, it is not known how many units would be needed to ensure, for example, a 5/6 HLA-A, B antigen and DRB1 allele-matched unit for the majority of patients of any race or ethnicity. Such projections, although complicated, are important in the consideration of the future funding needed for public UCB banks. A further issue is that of unit quality including: whether the critical determinants of a quality product; and how this should be regulated. McCullough et al. [35] investigated the quality of 268 units from banks in the United States and Europe and discovered that quality issues existed in 56% of units, with 10% likely and 35% potentially associated with patient risk. The major issues associated with UCB banking are discussed by Atlas [36] and Rubinstein [37].

Conclusion
UCB is a promising alternative HSC source although reaching its full potential will likely require a significant increase in the size of the global UCB inventory. If that can be achieved, this combined with measures such as improved preparative regimens, double-unit grafts, improved supportive care, measures to augment immune recovery will likely improve TRM and thus extend the adoption of UCBT to treat patients with high-risk hematologic malignancies.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest
Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 640).

11. This study represents the largest series of UCBT using NMA conditioning.
15. This is a landmark study demonstrating that UCB is an acceptable alternative HSC source for the transplantation of children with leukemia with comparable survival after 4–6/6 HLA-matched UCBT as compared with the gold standard of 8/8 allele-matched BM and superior survival after 6/6-matched UCBT.


This study is an excellent comprehensive review of the unique characteristics of immune reconstitution in UCBT and highlights the challenge of infectious complications in UCBT especially in the postengraftment period.


Factors associated with parameters of engraftment potential of umbilical cord blood

Thomas J. George, Michele W. Sugrue, Sarah N. George, and John R. Wingard

BACKGROUND: Umbilical cord blood (UCB) is an acceptable source of hematopoietic cells for transplantation with success being associated with the nucleated cell count (NCC), CD34+ cells, and colony-forming unit-granulocyte-macrophage (CFU-GM) content infused. A total of 1093 UCB samples with neonatal and parental characteristics that might influence hematopoietic content were examined.

STUDY DESIGN AND METHODS: UCB samples were screened, processed, and reevaluated for the above cell counts. These parameters of engraftment potential were analyzed for associations with neonatal and parental characteristics.

RESULTS: Postprocessed NCCs (median, 6.53 × 10^6 ± 2.80 × 10^6 SD; mean 7.30 × 10^6), CD34+ counts (median, 2.02 × 10^6 ± 2.20 × 10^6 SD; mean 2.65 × 10^6; r = 0.66; p < 0.001), and CFU-GM content (median, 2.85 × 10^3 ± 3.16 × 10^3 SD; mean, 3.54 × 10^3; r = 0.61; p < 0.001) all were strongly interrelated. Both initial volume (median, 77.6 ± 26.2 mL; SD, 81.9 mL) and initial NCC (median, 97.5 × 10^3 ± 4.90 × 10^3 SD; mean, 10.9 × 10^3) correlated well with postprocessed NCC (r = 0.80; r = 0.80; p < 0.01), CD34+ count (r = 0.40; r = 0.63; p < 0.01), and CFU-GM content (r = 0.38; r = 0.59; p < 0.01), with a stronger relationship seen with initial NCC. Infant birth weight (specifically, >5000 g), but not sex, gestational age, or cytomegalovirus status correlated strongly with collection volume and UCB cell counts. Units from minority volunteers contained relatively smaller volumes and hematopoietic content.

CONCLUSION: UCB banks should emphasize selecting the heaviest infants and processing large-volume units with high NCCs to optimize hematopoietic potential. Minority recruitment should be encouraged with consideration given to inherent racial differences in cell counts. There does not appear to be a significant relationship between other neonatal and parental characteristics and that of engraftment potential.

In recent years, partially matched umbilical cord blood (UCB) has been determined to be a suitable graft source for hematopoietic cell transplantation. Engraftment success, defined as the pace of neutrophil and platelet recovery and risk for transplant-related events, as well as overall transplant success, has been influenced by several variables including the underlying diagnosis, stage of the disease, and degree of donor-recipient HLA-match. The total nucleated cell count (NCC) infused, reported as cells per kg of recipient body weight, has been a reliable and reproducible indicator of outcome. The number of CD34+ cells and the number of colony-forming units-granulocyte-macrophages (CFU-GM) are also parameters used to assess hematopoietic potential of UCB units. Although the latter two parameters help to refine the hematopoietic potential of a graft, decisions as to processing and cryopreservation of freshly collected UCB units are generally made based on initial NCC and volume of cord blood because of the rapid availability of these parameters. Transplanted UCB grafts have been considered acceptable if they contain at least 1 × 10^6 to 3 × 10^6 NCCs per kg, with higher doses of cells infused correlating with shorter time to engraftment.

Although UCB is a rich source of hematopoietic stem and progenitor cells, UCB grafts contain much less volume, providing transplant recipients approximately 1/10th as many CD34+ cells compared to those receiving

ABBREVIATIONS: NCC(s) = nucleated cell count(s); UCB = umbilical cord blood.

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allogeneic marrow resulting in delayed hematopoietic recovery in recipients of UCB after transplantation. A high postthawing CD34+ content in UCB grafts has been shown to correlate with improved event-free survival in adult transplant recipients and may correlate with positive outcomes in the pediatric population. Unfortunately, the CD34+ content of thawed grafts is not consistently available at the time of transplant graft selection and is subject to reporting variability between cord banks.

Direct assessment of hematopoietic progenitor cells as determined in semisolid medium culture assays is helpful to predict graft hematopoietic potential as well as verifying viability of the cells after processing or cryopreservation. Recently, the dose of CFU-GM infused was found to better correlate with engraftment speed and risk for transplant-related events more accurately than NCC. Unfortunately, the use of these assays are limited by inconsistent growth and scoring between labs, which are potentially subject to technician experience and may take up to 14 days and therefore cannot be consistently used to determine graft adequacy before transplantation.

UCB banking has become established worldwide with funding provided by both public and private organizations. Limited resources require that UCB banks select, process, and store specimens with the greatest hematopoietic potential while maintaining a HLA-diverse population. Standardization in collection, processing, and storage between banks has been sought, particularly given it is given notable discrepancies between UCB banks and transplant centers.

We examined the characteristics of 1033 UCB samples collected at a single institution over a 2-year period. Our goal was to identify associations between the parameters of engraftment potential and a variety of neonatal and parental variables in an attempt to maximize future UCB specimen success.

**MATERIALS AND METHODS**

**Inclusion criteria**

Maternal donors were prospectively evaluated during their pregnancy, and appropriate medical information was voluntarily obtained after informed consent with institutional review board approval. Maternal serum was screened for infectious diseases including human immunodeficiency virus (HIV)-1 and -2, HIV-1 p24 antigen, hepatitis C antibody, hepatitis B core antibody, hepatitis B surface antigen, human T-lymphotropic virus-1 and -2, and cytomegalovirus (CMV). All positive test results except for CMV excluded the donor before collection. Gestational age of the infant at the time of delivery and UCB procurement was required to be at least 36 weeks.

**UCB collection**

Collection personnel with experience in venipuncture, infection control, and handling of biohazard material received training sufficient to perform the procedure. Immediately after either spontaneous vaginal or cesarean delivery, the umbilical cord was isolated and prepared in sterile fashion. UCB product collection was performed in the same manner for ex utero and in utero placental deliveries. Umbilical cords were clamped and the distal portion prepared for phlebotomy with a PVP iodophor scrub. Blood was aspirated into a 50-ml syringe containing 6 ml of citrate-phosphate-dextrose (Baxter Healthcare Corp., Deerfield, IL) via the umbilical vein. Once the syringe was filled, a new site was prepared and the process was repeated until no additional blood could be collected. Finally, all syringes were expressed into a 250-ml transfer pack. Participating facilities included Shands at the University of Florida, Gainesville, Florida; Shands at Alachua General Hospital, Gainesville, Florida; North Florida Regional Medical Center, Gainesville, Florida; Shands at Lake City, Lake City, Florida; and Baptist Health, Montgomery, Alabama.

**UCB processing and cryopreservation**

Collected UCB products were initially assessed for ABO/Rh, volume, and NCC. Products were considered ineligible for further manipulation if they contained fewer than $8 \times 10^9$ NCCs or less than 40 ml. Products containing $6 \times 10^9$ to $8 \times 10^9$ NCCs were processed and cryopreserved in toto. For products whose content exceeded $8 \times 10^9$ NCCs, HLA typing, sterility, and red cell (RBC) reduction procedures were performed. Briefly, 20 percent volume for volume hetastarch (Novaplus, Abbott Laboratories, North Chicago, IL) was added followed by centrifugation (5 min, 33 g). Cellular-rich supernatants were expressed into a 150-ml transfer pack (Baxter Healthcare Corp.) and recentrifuged (10 min, 226 g). Finally, the cellular-poor supernatant was expressed and the remaining pellet transferred to a cryopreservation container (Baxter Healthcare Corp.). Samples were taken for postprocessing analysis and the product was chilled in an ice bath. Cryoprotective solution containing 20 percent dimethyl sulfoxide (DMSO; Research Industries Corp., Salt Lake City, UT), 20 percent human serum albumin (Baxter Healthcare Corp.) in solution (Plasmalyte-A, Baxter Healthcare Corp.) was prepared to a volume equaling the RBC-reduced UCB product and chilled in an ice bath. Cryoprotective solution was slowly added to the cellular pellet, and containers were placed in aluminum cassettes. Freezing took place in a controlled-rate freezer according to standard protocol before storage in the vapor phase of liquid nitrogen.
Evaluation of the UCB products

Cell counts. NCCs were performed on the products before and after the processing procedure as detailed above. Counts were performed with a volume impedance cell counter (Sysmex F-800, Baxter Healthcare Corp.) employing lysing solution (Quicklyte, TOA Medical Electronics, Kobe, Japan) to lye RBCs as recommended by the manufacturer. All CD34+ and CFU-GM counts were analyzed after processing.

CD34+ cell content and viability. Modified flow cytometric analysis of samples was performed utilizing phycoerythrin–labeled anti-CD34 (Becton Dickinson Immunocytometry Systems, San Jose, CA) and peridinin chlorophyll protein–labeled anti-CD45 in bead-containing tubes (TruCount, Becton Dickinson). After RBC lysis with a fixative-free reagent, the impartant nucleic acid dye YO–PRO-1 (Molecular Probes, Eugene, OR) was added, and samples were analyzed with a four-color analysis (two lasers; FACSCalibur, Becton Dickinson). The main advantages of our CD34+ enumeration procedure are that it excludes nucleated RBCs and dead white blood cells (WBCs). It also directly enumerates CD34+ cells per μL, as opposed to providing a percentage of WBC that are CD34+. This procedure has previously been reported in detail for both CD34+ cell count enumeration and quantification of graft viability. NCC viability was estimated before banking by the trypan blue exclusion method (Gibco Laboratories, Grand Island, NY). A total of 100 nucleated cells were counted and a percentage of viable cells was determined.

Clonogenic assays. Clonogenic assays were performed on all processed UCB products. A total of 10^6 NCCs per 1-ml aliquot of methylcellulose with phytohemagglutinin–WBC-conditioned medium (StemCell Technologies, Inc., Vancouver, BC, Canada) were seeded in 35-mm petri dishes and incubated for 12 to 14 days at 37°C in a fully humidified atmosphere containing 5 percent CO2. Aggregates of at least 50 cells were scored for CFU-GM, CFU-granulocyte-erythrocyte-megakaryocyte-megakaryocytic (GEMM), or burst-forming units–erythroid with an inverted microscope. The total number of colonies for each product was then calculated based on the total NCC.

Data collection

Characteristics of the collected UCB were recorded in computer spreadsheet format (Microsoft Excel, Microsoft Corp., Seattle, WA). Specifically, data entry was performed on the following variables: initial volume of UCB collected, total initial NCC before processing, postprocessing volume before cryopreservation with or without RBC depletion, postprocessed NCC, CD34+, CFU-GM, and viability count. Gestational age, birth weight, infant sex, and maternal CMV status were obtained from the medical record at the time of the delivery. Voluntary self-reporting via survey completion of maternal and paternal race was provided before UCB harvest.

Statistical analysis

Data were analyzed with computer software (MINITAB 14, Minitab, Inc., State College, PA). Medians, standard deviations (SDs), and means were generated based on single variables as well as grouped by RBC manipulation, maternal race, paternal race, infant sex, and maternal CMV status. The Kruskal-Wallis test was used to detect differences between medians. Pearson correlation coefficients (r) were calculated with differences between coefficients determined by z-test or confidence intervals along with Fisher's r-to-z transformation. Two-by-two variables (CMV vs. race) were assessed with the Pearson chi-square test. Multivariate analysis calculations were performed with multivariate analysis of variance. All comparative tests were analyzed for significance at the 5 percent level of significance (i.e., p < 0.05).

Results

General characteristics of the 1035 UCB units are detailed in Table 1. CD34+ quantification and CFU-GM analysis were prospectively performed on all samples after processing. However, inconclusive results, contaminated samples, or technical difficulties resulted in reportable data in 965 (93%) and 1019 (99%) samples, respectively. RBC depletion was performed on 69 percent of samples. Maternal CMV status was available in 857 (87%) cases. Self-reporting of maternal and paternal race (n = 918 [89%] for both) revealed a predominance of Caucasian donors as volunteers. Infant sex was reported in 857 (83%) cases with a nearly equal distribution of sexes. Data were available on 580 (56%) and 556 (57%) samples regarding infant gestational age and birth weight, respectively.

Processing and RBC depletion

The initial NCC was reduced by approximately 33 percent after processing (Table 1). Of the initial samples collected, 68 percent contained enough NCC to undergo RBC depletion per protocol. Those samples contained a higher concentration of initial NCC, postprocessed NCC, CD34+, and CFU-GM content than those that did not undergo RBC depletion (p < 0.001 for each; Table 2). The process of RBC depletion resulted in a greater loss of NCC compared to those undergoing the standard processing procedure (37% reduction vs. 15%; p < 0.001, data not shown). The depleted samples retained an absolute larger concentration of hematopoietic precursors despite the added procedure of RBC depletion. Samples undergoing RBC depletion came from heavier infants (3624 ± 3462 g; p < 0.001). Otherwise, there was no dif-
difference between the need for RBC depletion and gestational age (p = 0.2) or CMV status (p = 0.7; data not shown).

Assessment of hematopoietic potential

The postprocessed NCC (median, 6.53 x 10^7 ± 2.80 x 10^6 SD; mean, 7.30 x 10^7) correlated strongly with CD34+ count (median, 2.02 x 10^6 ± 2.20 x 10^6 SD; mean, 2.85 x 10^6; r = 0.66; p < 0.001) and CFU-GM content (median, 2.05 x 10^3 ± 3.16 x 10^2 SD; mean, 3.54 x 10^3; r = 0.61; p < 0.001; Table 3). Likewise, a very strong correlation was seen between CD34+ and CFU-GM content (r = 0.69; p < 0.001). There was excellent internal correlation between the postprocessed NCC and the initial NCC collected (median, 9.75 x 10^7 ± 4.38 x 10^6 SD; mean, 10.9 x 10^7; r = 0.80; p < 0.001; Fig. 1). Both initial volume (median, 77.5 mL ± 26.2 mL SD; mean, 81.8 mL) and initial NCC correlated well with postprocessed NCC (r = 0.60; p = 0.001), CD34+ count (r = 0.40; p = 0.63; p = 0.01), and CFU-GM content (r = 0.38; p = 0.59; p = 0.01), with a stronger relationship seen with initial NCC.

Associations of neonatal and parental characteristics and preprocesing factors with engraftment potential parameters

Male infants were heavier by an average of 141 g (3852 g vs. 3511 g; p = 0.01) and had a higher content of CD34+ cells collected (2.25 x 10^8 vs. 1.96 x 10^8; p = 0.02).

---

### Table 1. UCB characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number (% of register)</th>
<th>Median</th>
<th>SD</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial volume (mL)</td>
<td>1033</td>
<td>77.5</td>
<td>26.2</td>
<td>81.3</td>
<td>40-193.5</td>
</tr>
<tr>
<td>Initial NCC (x10^6)</td>
<td>1033</td>
<td>9.75</td>
<td>4.88</td>
<td>10.9</td>
<td>4.42-28.5</td>
</tr>
<tr>
<td>Postprocessed volume (mL)</td>
<td>1033</td>
<td>50.0</td>
<td>33.4</td>
<td>65.2</td>
<td>20-222</td>
</tr>
<tr>
<td>Postprocessed NCC (x10^6)</td>
<td>1033</td>
<td>8.23</td>
<td>2.80</td>
<td>7.30</td>
<td>1.78-24.5</td>
</tr>
<tr>
<td>CFU-GM content (x10^9)</td>
<td>865 (93)</td>
<td>2.02</td>
<td>2.20</td>
<td>2.85</td>
<td>0.27-25.9</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>1019 (99)</td>
<td>2.09</td>
<td>3.18</td>
<td>3.54</td>
<td>0.07-92.8</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>868 (97)</td>
<td>35.9</td>
<td>1.1</td>
<td>36.2</td>
<td>35-42</td>
</tr>
</tbody>
</table>

* Includes 6 mL of anticoagulant.

### Table 2. Effect of processing and RBC depletion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Processed only (n = 973)</th>
<th>Processed with RBC depletion (n = 735)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial volume (mL)</td>
<td>79.5</td>
<td>87.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Initial NCC (x10^6)</td>
<td>8.60</td>
<td>11.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Postprocessed NCC (x10^6)</td>
<td>5.62</td>
<td>6.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD34+ cells (x10^6)</td>
<td>1.40 (n = 507)</td>
<td>2.49 (n = 436)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CFU-GM content (x10^9)</td>
<td>1.15 (n = 326)</td>
<td>3.41 (n = 269)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>35.0 (n = 170)</td>
<td>39.0 (n = 409)</td>
<td>0.2</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3362 (n = 171)</td>
<td>3624 (n = 414)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Medians are reported unless otherwise specified.

### Table 3. Correlations between UCB characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Initial volume</th>
<th>Initial NCC</th>
<th>Postprocessed NCC</th>
<th>CD34+</th>
<th>CFU-GM</th>
<th>Gestational age</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial NCC</td>
<td>0.71; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.80;</td>
<td>0.89;</td>
<td>0.70; &lt;0.001</td>
</tr>
<tr>
<td>Postprocessed NCC</td>
<td>0.80; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.80;</td>
<td>0.89;</td>
<td>0.70; &lt;0.001</td>
</tr>
<tr>
<td>CD34+</td>
<td>0.40; &lt;0.001</td>
<td>0.63; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.90;</td>
<td>0.89;</td>
<td>0.70; &lt;0.001</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>0.38; &lt;0.001</td>
<td>0.59; &lt;0.001</td>
<td>0.90; &lt;0.001</td>
<td>0.90;</td>
<td>0.89;</td>
<td>0.70; &lt;0.001</td>
</tr>
<tr>
<td>Gestational age</td>
<td>-0.26; 0.2</td>
<td>0.07; 0.06</td>
<td>0.05; 0.2</td>
<td>-0.13;</td>
<td>0.002</td>
<td>0.01; 0.8</td>
</tr>
<tr>
<td>Birth weight</td>
<td>0.25; &lt;0.001</td>
<td>0.20; &lt;0.001</td>
<td>0.17; &lt;0.001</td>
<td>0.14;</td>
<td>0.01;</td>
<td>0.10; &lt;0.001</td>
</tr>
</tbody>
</table>

* Data are reported as Pearson correlation coefficients (r); associated p value.
Fig. 1. Scatterplots of NCC correlations.

These cell count differences appeared to be dependent only on male sex, however, and not birth weight, upon multivariate analysis (p < 0.012; data not shown). Otherwise, infant sex did not influence volume collected, NCC, CFU-GM content, or gestational age.

Table 4 shows the relationship between gestational age and UCB characteristics. Gestational age (median, 39 ± 1.1 weeks SD; mean, 39.2 weeks; range, 38-42 weeks) did not correlate with volume or cell counts, except for a slight correlation between increased gestational age and lower CD34+ counts (r = -0.13; p = 0.002). As gestational age and birth weight appeared related (r = 0.24; p < 0.001), data were separated into one of three gestational age categories (<38, 38-40, and >40 weeks). There were significant differences in birth weight between young (<38 weeks) and average (38-40 weeks; 3199 g vs. 3596 g; p < 0.001) and young and older (>40 weeks) infants (3199 g vs. 3709 g; p < 0.001). Based on these age groups, however, there were no significant differences noted between volume collected, initial or postprocessed NCC, CD34+ cells, or CFU-GM content of the collected UCB.

Table 4 also shows the relationship between birth weight (median, 3596 g ± 427 g SD; mean, 3578 g; range, 2245-4898 g) and UCB characteristics. Small, but significant positive correlations were identified between birth weight and volume (r = 0.25; p < 0.001), initial NCC (r = 0.29; p < 0.001), postprocessed NCC (r = 0.17; p < 0.001), CD34+ count (r = 0.14; p < 0.001), and CFU-GM content (r = 0.18; p < 0.001). Data again were separated into one of three birth weight categories (<3000, 3000-4000, and >4000 g). Based on these groups, there were differences noted in initial volume, initial and postprocessed NCC, CD34+ cells, CFU-GM content, and gestational age. Specifically, the pattern was one of increased volume, cell count, and age per increase in birth weight category. The majority of these differences were significant, particularly as birth weight exceeded 3000 g (see Table 4 for details).

The characteristics of UCB samples examined by maternal and paternal race are shown in Table 5. Correlation coefficients between initial NCC, postprocessed NCC, CD34+, and CFU-GM content were similar for UCB collected from Caucasian or non-Caucasian parents (data not shown). There was a trend toward increased CD34+ cell content in UCB from Caucasian mothers (p = 0.06). Initial NCC and CFU-GM content were both significantly higher in samples obtained from Caucasian mothers compared to non-Caucasian mothers with no difference in
**TABLE 4. Effect of gestational age and birth weight**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number (%)</th>
<th>Volume (ml)</th>
<th>Initial NCC (x10^9)</th>
<th>Postprocessed NCC (x10^9)</th>
<th>CD34+ (x10^9)</th>
<th>CFU-GM (x10^9)</th>
<th>Weight (grams)</th>
<th>Birth weight (g)</th>
<th>Gestational age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;38</td>
<td>46 (9)</td>
<td>60.0</td>
<td>8.90</td>
<td>6.57</td>
<td>2.49</td>
<td>2.40</td>
<td>3129</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>38-40</td>
<td>47 (81)</td>
<td>79.6</td>
<td>10.10</td>
<td>6.58</td>
<td>2.08</td>
<td>2.48</td>
<td>3899</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>&gt;40</td>
<td>63 (11)</td>
<td>81.0</td>
<td>10.90</td>
<td>7.16</td>
<td>2.03</td>
<td>2.39</td>
<td>3769</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>p Value</td>
<td>0.8</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005‡</td>
</tr>
<tr>
<td>Weight (grams)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3000</td>
<td>51 (9)</td>
<td>72.5</td>
<td>8.12</td>
<td>8.20</td>
<td>2.18</td>
<td></td>
<td>39</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>3000-4000</td>
<td>442 (76)</td>
<td>78.1</td>
<td>9.59</td>
<td>8.65</td>
<td>4.00</td>
<td>2.48</td>
<td>39</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>&gt;4000</td>
<td>92 (16)</td>
<td>84.5</td>
<td>11.10</td>
<td>7.44</td>
<td>2.49</td>
<td>3.04</td>
<td>40</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>p Value</td>
<td>0.0011</td>
<td>0.0021§</td>
<td>0.0027**</td>
<td>0.02††</td>
<td>0.03††</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.005§§</td>
</tr>
</tbody>
</table>

* Medians are reported unless otherwise specified. Groups below refer to first, second, or third set of age or weight data.
† Range, 36-42 weeks.
‡ Range, 2345-4000 g.
§ p Value between Groups 1 and 2 and Groups 1 and 3; other NS (p = 0.06).
‖ Between Groups 1 and 2 (p = 0.05); Groups 1 and 3 (p = 0.004); Groups 2 and 3 (p = 0.077).
¶ Between Groups 1 and 2 (p = 0.06); Groups 1 and 3 (p = 0.004); Groups 2 and 3 (p = 0.02).
‖‖ Between Groups 1 and 2 (p = 0.02); Groups 2 and 3 (p = 0.001); other NS.
‖‖‖ Between Groups 1 and 2 (p = 0.00); Groups 2 and 3 (p = 0.05); other NS.
‖‖‖‖ Between Groups 1 and 2 (p = 0.001); Groups 1 and 3 (p = 0.001); Groups 2 and 3 (p = 0.01).

**TABLE 5. Racial differences in UCB characteristics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Maternal race</th>
<th>Paternal race</th>
<th>p Value</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caucasian</td>
<td>Non-Caucasian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial volume (ml)</td>
<td>76.1 (n = 81)</td>
<td>77.9 (n = 108)</td>
<td>0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Initial NCC (x10^9)</td>
<td>0.98</td>
<td>0.87</td>
<td>0.004</td>
<td>0.00</td>
</tr>
<tr>
<td>Postprocessed NCC (x10^9)</td>
<td>8.10</td>
<td>8.29</td>
<td>0.90</td>
<td>0.001</td>
</tr>
<tr>
<td>CD34+ cells (x10^9)</td>
<td>2.08 (n = 785)</td>
<td>1.85 (n = 105)</td>
<td>0.08</td>
<td>0.001</td>
</tr>
<tr>
<td>CFU-GM content (x10^9)</td>
<td>2.72 (n = 203)</td>
<td>2.61 (n = 104)</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>39.0 (n = 842)</td>
<td>39.0 (n = 842)</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3599 (n = 935)</td>
<td>3638 (n = 928)</td>
<td>3497 (n = 68)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Medians are reported unless otherwise specified.

Initial volume, gestational age, or birth weight. The difference noted in NCC was lost after processing (p = 0.06), however, thereby showing no significant difference between postprocessed NCC and maternal race.

Initial NCC and CFU-GM content were higher in samples obtained from Caucasian fathers compared to non-Caucasian fathers. There was also a significantly higher number of CD34+ cells in samples from Caucasian fathers (p = 0.03) as well as a larger initial volume (p = 0.01). Unlike in the maternal set, there remained persistent differences in NCC after processing (p = 0.01). There were no differences in gestational age, but infants from Caucasian fathers were heavier (3596 vs. 3497 g; p = 0.03).

CMV status and race were both determined in 826 samples (86%). There were significant differences between CMV status and maternal and paternal race. Specifically, 52 percent of Caucasian versus 65 percent of non-Caucasian mothers were CMV-positive (χ^2 = 5.0; p = 0.03), whereas 52 percent of Caucasian versus 68 percent of non-Caucasian fathers were CMV-positive (χ^2 = 9.6; p = 0.002). Regardless, CMV status did not impact volume, NCC, CD34+ cells, CFU-GM content, gestational age, or birth weight.

**DISCUSSION**

This analysis of data from our cord blood bank represents one of the largest regional databases reported in the literature with inclusion of laboratory, neonatal, parental, and racial data. Descriptive characteristics including volume and cell counts for this and other reported domestic and international registries are displayed in Table 6. Such a comparison is limited by a lack of standard collection and management policies between cord blood banks. Regardless, our data are consistent with other reported experiences in all categories, except CFU-GM content. Importantly, we note equivalent volumes collected, initial and postprocessed NCC, and CD34+ content. Progenitor cell viability in our unit was verified through the flow cytometric analysis of CD34+ cells as previously reported. Variability in CFU-determini

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nation has been previously reported, and our CFU-GM assays utilize techniques that differ from that reported by others. Specifically, our assays are read at 12 days (instead of 14 days), are not routinely RBC lysed for ease of viewing, and do not use recombinant growth factors in synthetic media. Taken together, these differences have the potential to underestimate our UCB CFU-GM content. As a result, our laboratory has changed CFU-GM assays in an attempt to become more consistent with other facilities; however, significant interinstitutional variability remains.

Both initial volume and initial NCC correlated well with postprocessed NCC (r = 0.69; r = 0.90; p < 0.01), CD34+ count (r = 0.40; r = 0.65; p < 0.01), and CFU-GM content (r = 0.38; r = 0.59; p < 0.01), with a stronger relationship seen with initial NCC. Others have reported initial volume collected to be an accurate and reliable measure of CD34+ count. Our data suggest that initial NCC is a better surrogate marker than volume for UCB hematopoietic potential. Strong correlations were also identified between postprocessed NCC and CD34+ (r = 0.56) and with CFU-GM content (r = 0.61). Both were highly significant and consistent with correlations previously published (NCC/CD34+ range, 0.59-0.87). NCC/CFU-GM range, 0.49-0.89). CD34+ and CFU-GM content also had a strong direct correlation (r = 0.68), suggesting that CD34+ content may predict CFU-GM content better than total NCC, consistent with the reporting of others.

As a measure of internal control, there was a very strong correlation between postprocessed and initial NCC (r = 0.9; p < 0.001) despite the degree of RBC depletion and processing required. Indeed, the procedure of UCB processing results in the loss of approximately one-third of NCC, consistent with that previously reported (35%). The majority of this loss occurs in those samples that undergo RBC depletion (37% vs. 15%; p < 0.001), which represents the majority (88%) of our samples. Despite this increased loss, however, those samples with higher initial NCC (which subsequently undergo RBC depletion) retain an adequate postprocessed NCC. RBC depletion of UCB limits the volume of specimens required for storage and DMSO concentration, allows uniform and rapid freezing and thawing, lessens the potential exposure of mismatched RBC antigens to transplant recipients, and minimizes free hemoglobin infused. Although not internationally standardized, our UCB bank required a minimum NCC of 6 x 10^6 before processing and storage, but has recently increased this number to 8 x 10^6. It would appear that our threshold of performing RBC depletion based on initial NCC of greater than 8 x 10^6 is justified. Our units were not assessed for the nucleated RBC content of the NCC, however. It has been suggested that nucleated RBCs can comprise a significant proportion of the NCC in a graft and lyse more easily during processing and thawing. It is possible that such loss of NCC and nucleated RBCs in processing could adversely affect the speed of myeloid engraftment after transplantation.

As should be expected, there was no significant relationship between infant sex and most UCB characteristics. Male infants in our registry were heavier than their female counterparts, with a clear relationship established between increased birth weight and UCB cell content as a possible explanation (see Table 4 and text below). Male sex alone, however, appeared to be the major influence on these differences, as suggested upon multivariate analysis. Although only 57 percent of our data set contained birth weight information, our findings are consistent with those of others who reported an independent association between increased cells counts and male sex, particularly with regard to the CD34+/CD61 subset. Perhaps there may be an underlying effect of in utero sex-specific hormones on neonatal circulation and hematopoiesis.

All infants were of gestational age between 36 and 42 weeks. Newborns of increasing gestational age were bigger, as expected, but had no significant differences as related to cell counts, although a negative correlation was identified with CD34+ counts. Inverse relationships
ACKNOWLEDGMENTS
The authors thank the volunteers and donors of UCB at each of the locations previously specified whose selfless contributions are truly deserving. Additionally, we are indebted to J.A. Iturraspe and G.A. Martinez of Life South Community Blood Centers for their acquisition and processing of samples as well as D.D. Fisk, C.E. Hutchison, T.D. Peña, E.H. Rosenau, and C.C. Roberts at Shands Hospital, who all provided additional processing of samples, cell counting, and data collection. Finally, we thank J.C. Hopkins for data entry and database management.

REFERENCES

Cels利用 A. Rodrigues, Guillermo Sants, Claudia G. Brunstein, Jaime Sants, John E. Wagner, Marc Renaud, Marcos de Lima, Mitchell S. Cairo, Sabine Fürst, Bernard Rio, Christopher Daley, Enric Carreras, Jean-Luc Harousseau, Mohamad Mohsy, Denis Taveira, Peter Dreger, Anna Sureda, Eliane Glickman, and Vanderzon Rocha

ABSTRACT

Purpose
To determine risk factors of umbilical cord blood transplantation (UCBT) for patients with lymphoid malignancies.

Patients and Methods
We evaluated 104 adult patients (median age, 41 years) who underwent unrelated donor UCBT for lymphoid malignancies. UCB grafts were two-antigen human leukocyte antigen–mismatched in 68%, and were composed of one (n = 78) or two (n = 26) units. Diagnoses were non-Hodgkin’s lymphoma (NHL, n = 61), Hodgkin’s lymphoma (HL, n = 29), and chronic lymphocytic leukemia (CLL, n = 14), with 87% having advanced disease and 60% having experienced failure with a prior autologous transplant. Sixty-four percent of patients received a reduced-intensity conditioning regimen and 46% low-dose total-body irradiation (TBI). Median follow-up was 18 months.

Results
Cumulative incidence of neutrophil engraftment was 84% by day 60, with greater engraftment in recipients of higher CD34⁺ kg/cell dose (P = .0004). CI of non-relapse-related mortality (NRM) was 28% at 1 year, with a lower risk in patients treated with low-dose total-body irradiation (TBI); P = .03. Cumulative incidence of relapse or progression was 31% at 1 year, with a lower risk in recipients of double-unit UCBT (P = .03). The probability of progression-free survival (PFS) was 40% at 1 year, with improved survival in those with chemosensitive disease (49% vs 34%; P = .03), who received conditioning regimens containing low-dose TBI (60% vs 23%; P = .001), and higher nucleated cell dose (49% vs 21%; P = .009).

Conclusion
UCBT is a viable treatment for adults with advanced lymphoid malignancies. Chemosensitive disease, use of low-dose TBI, and higher cell dose were factors associated with significantly better outcome.

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Allogeneic hematopoietic stem-cell transplantation (HSCT) is a curative approach for patients with advanced, relapsed, or refractory non-Hodgkin’s lymphoma (NHL), Hodgkin’s lymphoma (HL), and chronic lymphocytic leukemia (CLL). Comparative studies have reported lower relapse rates after allogeneic transplant relative to autologous transplant. However, conventional allogeneic HSCT is associated with high non-relapse-related mortality (NRM), which offsets the potential survival benefit of this procedure. Reduced-intensity conditioning (RIC) regimens have been used with increasing frequency in such high-risk populations. Low relapse rates after RIC transplant suggest that the graft-versus-lymphoma (GVL) effect of donor T cells is retained. Umbilical cord blood (UCB) is an alternative source of hematopoietic stem cells for the treatment of hematologic malignancies in patients lacking a
human leukocyte antigen (HLA)-matched donor. Advantages of UCB include prompt availability and decreased risk of graft-versus-host disease (GVHD) despite HLA mismatch. These attributes make UCB applicable to nearly all patients, particularly those with less common tissue types, such as those in ethnic and racial minorities. However, the low number of progenitor cells has been associated with delayed engraftment and increased risk of NRM. Strategies to overcome this barrier include the use of two partially HLA-matched UCB units (double UCBT). There have been a few isolated reports for refractory NHL and malignant lymphoma treated by RIC-UCBT. This larger analysis has allowed us to report the general experience of unrelated UCBT in the treatment of advanced lymphoid malignancies in adults, and to identify treatment- and disease-based factors associated with better or poorer outcomes.

**Data Collection**

Euroword is a registry of related and unrelated UCBT that works in collaboration with the European Group of Blood and Marrow Transplantation (EBMT), and Netcord banks. Netcord is an international organization that encompasses cord blood banks all over the world, mostly in Europe (the Appendix, online only, contains a listing of banks). Netcord and EBMT databases provided data on UCBT. Centers not associated with EBMT were asked to complete reports if UCB units were obtained from Netcord banks. All data were verified and updated by the institution’s physicians and data managers. All patients or legal guardians provided informed consent for the UCBT according to the Declaration of Helsinki.

**Inclusion Criteria**

The study included patients with malignant lymphoma (both HL and NHL) or CLL (1) who received an unrelated and unmanipulated single-unit or double-unit UCBT; (2) who were older than 15 years at the time of transplantation; and (3) for whom there were adequate and sufficient data to perform the analysis. Twelve patients included in this study were previously reported.

**End Point Definitions**

The primary end point was progression-free survival (PFS) at 1 year, defined as the time from transplantation to relapse, disease progression, or death. Other end points included incidence of neutrophil recovery, defined as first of 3 consecutive days with a neutrophil count of at least 0.5 x 10^9/L, and the incidence of platelet recovery as the first of 7 consecutive days of an unsupported platelet count of at least 20 x 10^9/L; graft failure was defined as no sign of neutrophil recovery, as well as transient engraftment of donor cells 60 days after transplantation; acute GVHD at day 100 and chronic GVHD at 1 year, diagnosed and graded according to published criteria, with histopathologic confirmation when possible; relapse or progression at 1 year, as defined by the criteria on the basis of clinical, imaging, or laboratory evidence; and NRM at 6 months and at 1 year, defined as deaths related to transplantation and not to relapse. Chimerism data was evaluated in the first 3 months after UCBT. Full donor chimerism was defined as the presence of more than 95% of the cells of donor origin, mixed chimerism if more than 5% and less than 95% of donor cells, and autologous recovery if less than 5% of donor cells. Data on the method of chimerism detection were not collected.

**Statistical Analysis**

Data were analyzed through March 2007. Cumulative incidence function (CIF) using death as a competing event was used to estimate neutrophil and platelet engraftment, acute and chronic GVHD, NRM, and relapse. The Kaplan-Meier method was used to estimate overall survival (OS) and PFS. For continuous variables, the median was used as the cutoff point. For assessment of prognostic factors using CIF, univariate and multivariate analyses were performed using the Gray’s test and the proportional subdistribution hazard regression model of Fine and Gray. For OS and PFS, log-rank tests and Cox proportional-hazards model in univariate and multivariate analyses were used. Acute and chronic GVHD were assessed as time-dependent covariates for PFS. Each potential risk factor was tested independently. All factors that reached P ≤ .05 in the univariate analysis were included in the multivariate model. All models were built using a forward stepwise method. Only factors that reached a P ≤ .05 were held in the final model. Of note, the factors "lymphoma subtype" and "use of TBI" were initially classified into multiple categories. However, in an effort to minimize multiple comparisons, and as there were no statistical differences between the categories "no TBI" and "high-dose TBI" (Appendix Table A1, online only), these categories were collapsed and the variable "use of TBI" was analyzed as "low-dose TBI versus others." The variable "lymphoma subtype" was not included in the final multivariate analysis because the group of patients with mantle-cell lymphoma was too small, and clinically different from indolent lymphoma. The use of antithymocyte or antithymocyte globulin (ATG/ALG) was also not included in the final model because of a strong correlation with myeloablative conditioning regimen (Appendix Table A3, online only). Statistical analyses were performed using the statistical software package SPSS.
performed with SPSS (SPSS Inc, Chicago, IL), and S-Plus (Insightful Corp, Seattle, WA) software packages.

**Patient and Disease Characteristics**

A total of 104 patients from 34 EBMT transplant centers and 14 non-EBMT centers, who underwent transplantation between January 1996 and June 2007, met the inclusion criteria: 15 patients received transplants from 1996 to 2001, 30 from 2002 to 2004, and 59 from 2005 to 2007. Sixty-one patients had NHL, 29 had HL, and 14 CLL. Patient and disease characteristics are summarized in Table 1. Forty-two patients with a response to the last therapy before the transplant (complete or partial remission) were considered chemosensitive, and 62 patients with primary refractory disease or refractory relapse before transplant were considered chemoresistant.

**Graft and Transplant Characteristics**

Graft and conditioning regimen characteristics are summarized in Table 2. A total of 78 patients received a single UCBT, and 26 received a double UCBT.

<table>
<thead>
<tr>
<th>Table 1. Patient and Disease Characteristics (N = 104)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>Age at transplantation, years</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Weight at transplantation, kg</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Recipient CMV positive</td>
</tr>
<tr>
<td>Histology at diagnosis (WHO classification)</td>
</tr>
<tr>
<td>Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>Mature B-cell neoplasms</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
</tr>
<tr>
<td>Small lymphocytic lymphoma</td>
</tr>
<tr>
<td>Mature T-cell neoplasms</td>
</tr>
<tr>
<td>Peripheral T-cell lymphoma</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma</td>
</tr>
<tr>
<td>Extramedullary NK/T-cell lymphoma</td>
</tr>
<tr>
<td>Angioimmunoblastic T-cell lymphoma</td>
</tr>
<tr>
<td>Hepatosplenic T-cell lymphoma</td>
</tr>
<tr>
<td>Subcutaneous panniculitis-like T-cell lymphoma</td>
</tr>
<tr>
<td>Interval between diagnosis and transplant, months</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Prior autologous transplant</td>
</tr>
<tr>
<td>Disease status at UCBT</td>
</tr>
<tr>
<td>Complete remission</td>
</tr>
<tr>
<td>Partial remission</td>
</tr>
<tr>
<td>Refractory disease or relapse</td>
</tr>
</tbody>
</table>

Abbreviations: CMV, cytomegalovirus; NK, natural killer; UCBT, umbilical cord blood transplantation.

<table>
<thead>
<tr>
<th>Table 2. Graft and Transplant Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic</td>
</tr>
<tr>
<td>No. of UCB units</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>No. of HLA disparities*</td>
</tr>
<tr>
<td>6/6 match</td>
</tr>
<tr>
<td>5/6 match</td>
</tr>
<tr>
<td>4/6 match</td>
</tr>
<tr>
<td>3/6 match</td>
</tr>
<tr>
<td>No. of HLA disparities†</td>
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<tr>
<td>2 units 6/6 match</td>
</tr>
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<td>2 units 5/6 match</td>
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<td>2 units 4/6 match</td>
</tr>
<tr>
<td>1 unit 5/6 and 1 unit 4/6 match</td>
</tr>
<tr>
<td>1 unit 4/6 and 1 unit 3/6 match</td>
</tr>
<tr>
<td>No. of total nucleated cells infused, ×10⁹/kg</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>No. of total CD34+ cells infused, ×10⁹/kg</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Conditioning regimen (n = 100)</td>
</tr>
<tr>
<td>Reduced-intensity</td>
</tr>
<tr>
<td>Cyclophosphamide + fludarabine + TBI 2 Gy</td>
</tr>
<tr>
<td>Busulfan + thiotepa + fludarabine</td>
</tr>
<tr>
<td>Cyclophosphamide + fludarabine ± thiotepa</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Myeloablative</td>
</tr>
<tr>
<td>Busulfan + thiotepa + fludarabine</td>
</tr>
<tr>
<td>Busulfan + cyclophosphamide ± thiotepa ± melphalan</td>
</tr>
<tr>
<td>Cyclophosphamide + TBI 12 Gy ± fludarabine</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Use of total body irradiation</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Low-dose</td>
</tr>
<tr>
<td>High-dose</td>
</tr>
<tr>
<td>Graft-versus-host disease prophylaxis (n = 100)</td>
</tr>
<tr>
<td>Cyclosporin + mycophenolate mofetil</td>
</tr>
<tr>
<td>Cyclosporin + prednisone</td>
</tr>
<tr>
<td>Cyclosporin ± methotrexate</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Use ATG or ALG (n = 102)</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Follow-up time for survivors, months</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Range</td>
</tr>
</tbody>
</table>

Abbreviations: UCB, umbilical cord blood; HLA, human leukocyte antigen; TBI, total body irradiation; ATG, antithymocyte globulin; ALG, antithymocyte globulin.

*One unit, antigen-level HLA-A and B and allele-level HLA-DRB1 typing.
†Two units, antigen-level HLA-A and B and allele-level HLA-DRB1 typing.
Conditioning regimen varied according to the transplant center. A total of 64 patients received an RIC regimen, and 36 received a myeloablative conditioning regimen. For four patients, detailed data on the conditioning regimen were not available. Median follow-up time for survivors was 18 months (range, 3 to 74 months).

**Engraftment and Chimerism Studies**

The cumulative incidence of neutrophil recovery was 84% by day 60. Neutrophil recovery occurred in 86% of patients at a median of 17 days (range, 3 to 54 days) for patients who received RIC and in 83% at a median of 22 days (range, 11 to 48 days) for patients who received myeloablative regimens. Eight patients died before day +30 without achieving neutrophil engraftment. Primary graft failure occurred in nine patients: five patients had autologous reconstitution and four engrafted after a second transplant (two patients received an autograft; one a UCET and one a peripheral blood-stem-cell transplant).

In a univariate analysis, the following variables were associated with a higher incidence of neutrophil engraftment (Table 3): use of low-dose TBI in the conditioning regimen (92% v 73% for patients not receiving TBI and 87% for patients receiving high-dose TBI; \( P = .0007 \), regimens not incorporating ATG/ALG (91% v 76%,

### Table 3. Univariate Analysis for Outcomes After Umbilical Cord Blood Transplantation for Patients With Lymphoid Malignancies (\( N = 104 \))

<table>
<thead>
<tr>
<th>Variable</th>
<th>Neutrophil Engraftment at Day 60 (( N = 84 ))</th>
<th>Non-Relapse-Related Mortality at 1 Year (( N = 28 ))</th>
<th>Acute Graft-Versus-Host Disease at Day 100 (( N = 24 ))</th>
<th>Progression-Free Survival at 1 Year (( N = 40 ))</th>
<th>Overall Survival at 1 Year (( N = 48 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>54</td>
<td>67</td>
<td>38</td>
<td>12</td>
<td>34</td>
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<tr>
<td>≥ 41</td>
<td>49</td>
<td>62</td>
<td>19</td>
<td>38</td>
<td>27</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>.04</td>
<td>.002</td>
<td>NS</td>
<td>.02</td>
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<tr>
<td>Lymphoma subtype</td>
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<tr>
<td>Indolent NHL</td>
<td>26</td>
<td>65</td>
<td>20</td>
<td>36</td>
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<tr>
<td>Mantle cell lymphoma</td>
<td>8</td>
<td>63</td>
<td>0</td>
<td>38</td>
<td>26</td>
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<td>Hodgkin’s lymphoma</td>
<td>29</td>
<td>90</td>
<td>35</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td>Aggressive NHL</td>
<td>41</td>
<td>65</td>
<td>34</td>
<td>22</td>
<td>37</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>.02*</td>
</tr>
<tr>
<td>Disease features</td>
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<td></td>
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<td>NS</td>
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</table>

**NOTE.** Superscripted parentheses refer to the \( P \) value for pairwise tests: (1) \( v < 2 \); (2) \( v < 4 \); (3) \( v < 4 \); (4) \( v > 4 \). (1) and (2) \( v < 3 \) and (4) is .002; (1) \( v > 3 \) is .30; (2) \( v \) (1 and 3) is < .0001. Abbreviations: NS, not significant; NHL, non-Hodgkin’s lymphoma; UCB, umbilical cord blood; RIC, reduced-intensity conditioning; MAC, myeloablative conditioning; TBI, total-body irradiation; ATG, antithymocyte globulin; ALG, antilymphocyte globulin; TNC, total nucleated cells; NS, not significant. *\( P \) of.
P = .004), and infused CD34+ cell dose greater than 1.0 × 10^6/kg (96% vs 77%; P < .0001). In a multivariate analysis, the use of low-dose TBI (P = .04; Table 4), and a higher CD34+ cell dose (P = .0004) remained favorably associated with engraftment. Number of HLA mismatches was not identified as a factor associated with neutrophil engraftment.

The cumulative incidence of platelet engraftment was 65% by day 180. In a univariate analysis, factors associated with higher incidence of platelet engraftment were use of low-dose TBI (89% vs 53% in patients not receiving TBI and 47% in those receiving high-dose TBI; P < .0001), regimens not incorporating ATG/ALG (71% vs 57%; P = .04), and infused CD34 cell dose greater than 1.0 × 10^6/kg (85% vs 58%; P = .002). In a multivariate analysis, only low-dose TBI remained associated with platelet engraftment (P = .003).

In recipients of single UCBT, chimera studies were available for 54 of 62 assessable patients. Forty patients (74%) had complete chimera, and eight patients (15%) had mixed chimera at first testing (before day +100). Of these, four patients became complete chimeras at the second or third evaluation.

In recipients of double UCBT, chimera data were available in 17 out of 21 assessable patients. Sixteen patients (94%) had complete chimera and one patient (6%) had a mixed chimera. In 16 cases, engraftment was derived from one unit and in two cases, from both units.

NRM

Twenty-nine patients died as a result of non-relapse-related causes. The principal causes of NRM were infection (69%): bacterial (n = 9), viral (n = 6), or fungal (n = 5). Cumulative incidence of NRM was 24% at 6 months and 28% at 1 year. Factors associated with a lower NRM were age at least 41 years (19% vs 38%; P = .04), use of low-dose TBI (13% vs 50% in patients not receiving TBI and 20% in those receiving high-dose TBI; P = .0006), regimens not incorporating ATG/ALG (18% vs 38%; P = .04), and total nucleated cell (TNC) dose higher than 2 × 10^7/kg (22% vs 41%; P = .02). In a multivariate analysis, the use of low-dose TBI (P = .03), and a TNC dose higher than 2 × 10^7/kg (P = .045) were associated with lower NRM. Although patients who received RIC also tended to have lower NRM compared with those receiving myeloablative regimens (20% vs 38%), this beneficial effect was driven only by RIC regimens incorporating low-dose TBI, and not by the others.

GVHD

The cumulative incidence of acute GVHD grades 2 to 4 and 3 to 4 was 24% and 8%, respectively. Factors associated with a higher risk of acute GVHD were age 41 years or older (38% vs 12%; P = .002), use of low-dose TBI (39% vs 11% in patients not receiving and 13% in those receiving high-dose TBI; P = .003), regimens not incorporating ATG/ALG (33% vs 14%; P = .02), and RIC-UCBT (32% vs 14%; P = .04). In a multivariate analysis, only older age remained significantly associated with the risk of acute GVHD (P = .02).

Fifty-two patients were assessable for chronic GVHD; the cumulative incidence at 1 year was 18%. Eight patients (15%) developed limited and 10 (19%) extensive chronic GVHD.

Relapse or Progression

The cumulative incidence of relapse or progression was 31% at 1 year and 35% at 2 years. Overall, 35 patients (33%) relapsed or progressed after the UCBBT, with a median time to relapse or progression of 5 months (range, 1 to 33 months). Of these 35 patients, 29 (83%) were transplanted in relapse, partial remission, or had refractory disease at transplant.

Factors associated with lower relapse or progression rates were chemoresensitive disease (22% vs 38%; P = .05) and use of double UCBBT (13% vs 38%; P = .009). In a multivariate analysis, only the use of double UCBBT (P = .02) remained associated with lower relapse risk.

PFS and OS

The probability of PFS was 40% at 1 year and 36% at 2 years. Factors associated with PFS were age at least 41 years (54% vs 28%; P = .02), presence of chemoresistant disease (49% vs 34%; P = .04), histologic subtype (60% in indolent NHL, 75% in mantle cell NHL, 29% in aggressive NHL, and 30% in HL; P = .02; Fig 2), use of low-dose TBI (59% vs 20% in patients not receiving TBI and 33% in those receiving high-dose TBI; P < .0001), use of regimens not incorporating ATG/ALG (56% vs 23%; P = .001; Fig 3), and a TNC dose higher than 2 × 10^7/kg (49% vs 21%; P < .0001). In a multivariate analysis, use of low-dose TBI (P = .001), chemoresistant disease (P = .03), and a TNC dose higher than 2 × 10^7/kg (P = .009) remained factors associated with a better PFS.

Acute or chronic GVHD, analyzed as time dependent covariates, were not statistically associated with PFS (for acute GVHD, relative risk [RR] = 0.56; 95% CI, 0.56 to 1.11; P = .10; for chronic GVHD, RR = 0.39; 95% CI, 0.09 to 1.73; P = .22).

OS at 1 year was 48%. Factors associated with OS were similar to those for PFS: older age (62% vs 35%; P = .02), use of low-dose TBI (74% vs 20% in patients not receiving TBI and 39% in those receiving high-dose TBI; P < .0001), use of regimens not incorporating ATG/ALG (68% vs 26%; P < .0001), and higher UCBBT graft TNC dose greater than 2 × 10^7/kg (61% vs 22%; P < .0001). In multivariate analysis, use of low-dose TBI (P < .0001), and TNC dose higher than 2 × 10^7/kg (P = .01) remained associated with better OS. In the subgroup of patients with indolent lymphoid disease, PFS was 75% in patients with follicular lymphoma and 43% in those with CLL.

### Table 4. Multivariate Analysis for NRM, Relapse or Progression, PFS, and OS

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative Risk</th>
<th>95% CI</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Neutrophil engraftment</td>
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<td></td>
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<tr>
<td>Use of low-dose TBI</td>
<td>1.02</td>
<td>1.03 to 2.57</td>
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<td>CD34+ cells &gt; 1 × 10^6/kg</td>
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<tr>
<td>Use of low-dose TBI</td>
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<td>0.10 to 0.89</td>
<td>.03</td>
</tr>
<tr>
<td>TNC &gt; 2 × 10^7/kg</td>
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<td>0.21 to 0.98</td>
<td>.045</td>
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<td></td>
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<td>2 UCB units</td>
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<td>0.28 to 0.83</td>
<td>.01</td>
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</table>

Abbreviations: NRM, non-relapse-related mortality; PFS, progression-free survival; OS, overall survival; TBI, total-body irradiation; TNC, total nucleated cell; GVHD, graft-versus-host disease; UCB, umbilical cord blood.
In the subgroup of patients who were not in complete remission at transplant (n = 80), 30 (38%) remain in remission after UCBT with a median follow-up of 18 months (range, 4 to 57 months). PFS and OS at 1 year were 45% and 48%, respectively. PFS was 69% for patients who received low-dose TBI versus only 9% in those not receiving TBI and 36% in those who received high-dose TBI (P < .0001).

In the present study, we demonstrated that UCBT is a viable option for patients with lymphoma and CLL. Despite the fact that most patients received transplants in an advanced phase of their disease, relatively low NRM and good survival rates were observed. Especially favorable characteristics were chemosensitive disease, use of low-dose TBI, and higher cell doses.

To date, there have been only a few isolated reports on the use of UCBT in patients with advanced lymphoid malignancy.26-49 The use of conventional allogeneic HSCT in patients with lymphoma and CLL is still limited.29 The reported studies are heterogeneous in terms of patient, transplant, and disease features, which make comparisons difficult.

Our results, using unrelated donor UCBT, are comparable to those using HLA-matched donors.22,25,44-47 We observed an NRM incidence of 28% and PFS and OS rates of 40% and 48% at 1 year, respectively. Branon et al29 observed 20% of NRM of and a PFS of 59% at 14 months (median follow-up time) in 38 patients with advanced lymphoma who received an RIC HLA-matched sibling donor transplant. The Lymphoma Working Party of the EBMT reported a NRM of 26% and a PFS of 46% at 1 year with a median follow-up of 7 months, in 183 patients with lymphoma who received an RIC-HSCT.22 Survival was significantly better in those with chemosensitive disease, HL, and indolent NHL.

In the present study, chemosensitivity also favorably influenced PFS (49% vs 34%), and OS (54% vs 44%). Besides, we also observed that patients with indolent NHL presented a significantly better outcome: NRM, PFS, and OS rates were 20%, 60%, and 68%, respectively. A better response rate in indolent disease is expected in this group of patients in which RIC regimens were the most frequently used. Besides, the observed worse prognosis of UCBT for both HL and aggressive NHL might also be related to the high toxicity of the conditioning regimen, yielding a high NRM rate, and a high relapse risk in a group of patients with advanced phases of disease because UCBT is usually the last possibility of treatment and is still considered experimental by many transplant centers.

To our knowledge, this is the first study to report patients with CLL who received a UCBT. We observed a 1-year PFS and OS of 43% and 51%, respectively. These results are comparable with those of allogeneic HSCT in the RIC setting, with PFS rates ranging from 34% to 52% and OS from 51% to 69%.46-50

We observed a significantly lower NRM and better PFS and OS rates in patients who received low-dose TBI. The assumption is that this regimen provides sufficient immunosuppression with lower risk of regimen-related toxicity, thus accounting for its overall beneficial effect. RIC regimens not incorporating low-dose TBI resulted in outcomes comparable to that of myeloablative therapies. The GVL effect appears to be sufficient after low-dose TBI, on the basis of the observed risks of relapse and progression in this series.

Immunosuppression with ATG/ALG was associated with poor outcomes in a univariate analysis. However, because of the correlation with myeloablative conditioning regimens in the majority of cases in our series, the role of ATG/ALG was not appropriately addressed and should be further evaluated in a more homogenous population.

In this multicentric based-registry analysis, we were not able to analyze the association of center effect with outcomes because of the small number of patients included per center and the changes over time of the conditioning regimens, even in a same center.

One of the intriguing findings of this study is the possible enhanced GVL effect associated with double UCBT. Such a finding also has been observed in adults with various hematologic malignancies.51,52 Whether this apparent enhancement of GVL is simply the result of a greater state of alloimmune immune cell activation or the greater use of more HLA-disparate UC B units has yet to be determined.

Incidence of acute GVHD was higher in patients older than 41 years, but age was not associated with PFS. One could argue that this observation could be related to a stronger GVL effect. However, there was no statistical association between GVHD and PFS, despite a trend of improved PFS in patients presenting GVHD. The GVL effect after UCBT in patients with lymphoma needs to be analyzed in a larger series of patients and with a longer follow-up.

In conclusion, UCBT is a viable alternative in adult patients with advanced lymphoma and CLL who lack an HLA-matched donor, with particularly encouraging results for patients with chemosensitive disease receiving low-dose TBI-based conditioning regimens and adequate cell doses. On the basis of our findings, several important strategies should be considered: (1) greater use of less toxic RIC regimens, such as those containing low-dose TBI, (2) better selection of UC B units, and (3) broader use of double UC BT.

The authors indicated no potential conflicts of interest.

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Unrelated cord blood transplants in adults with hematologic malignancies

Background and Objectives. We analyzed outcomes and risk factors after unrelated cord blood transplantation (CBT) in adults with hematologic malignancies.

Design and Methods. One hundred and seventy-one patients were transplanted after 1997. Their median age was 29 years (15-55), and the median follow-up time was 18 months (2-71). Most patients had acute or chronic leukemia (n=142, 83%), 91 (53%) were transplanted in advanced phase and an autologous transplant had failed in 32 (19%). Most patients (87%) received an HLA-mismatched cord blood unit with 1-2 HLA disparities. At infusion, the median number of nucleated cells and CD34+ cells was 2.1x10^6/kg and 1x10^5/kg, respectively.

Results. The cumulative incidence of neutrophil recovery at day 60 was 72±3% with a median of 28 days (11-57). A higher neutrophil count and use of hematopoietic growth factors were independently associated with faster neutrophil recovery. The cumulative incidence of grade III-IV acute graft-versus-host disease was 32±4% and this complication was not associated with the number of HLA mismatches. The 2-year cumulative incidence of chronic graft-versus-host disease, transplant related mortality and relapse were 36±10%, 51±4% and 22±4%, respectively. At 2-years, disease-free survival for patients transplanted in early, intermediate and advanced phases of disease was 41±9%, 34±10% and 18±4%, respectively. In multivariate analyses, advanced disease status was an adverse factor for relapse and disease-free survival.

Interpretation and Conclusions. Unrelated CBT is a clear alternative for adults with hematological malignancies lacking an HLA-matched related or unrelated donor. The choice of units containing a higher neutrophil count and a policy of earlier transplantation are likely to provide better results.

Key words: unrelated cord blood transplants, adults, hematologic malignancies.

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Umbilical cord blood from unrelated donors represents a clear alternative source of hematopoietic progenitor cells to bone marrow for children lacking an HLA identical sibling. The lower risk of graft-versus-host disease (GVHD) in the cord blood transplant setting than in bone marrow transplants permits less stringent criteria for donor-recipient HLA matching. Moreover, cord blood units are acquired faster than bone marrow from unrelated donors, which is particularly relevant for patients who require an urgent transplant. However, despite the high proliferative potential of cord blood hematopoietic progenitors, the lack of nucleated and CD34+ cells in a single cord blood unit has represented the major limitation to the use of unrelated cord blood transplantation (CBT) in adults, due to concern about the risk of graft failure and delayed engraftment. However, pilot studies, including small series of adults transplanted from unrelated, mostly HLA mismatched, cord blood donors, reported encouraging results in terms of engraftment, incidence of GVHD, risk of relapse and event-free survival. As CBT has been increasingly used for adults, two independent studies have recently compared the results of unrelated CBT and bone marrow transplantation in adult patients with acute leukemia. Despite some differences between the two analyses, in both studies the main outcomes (relapse, transplant-related mortality, leukemia-free survival) were similar in the patients receiving the two different types of transplants.

Herein, we report a retrospective analysis of results and risk factors for different outcomes in 171 unrelated cord blood transplants performed between January 1998 and January 2005 in adults with hematologic malignancies and reported by 69 centers to the Eurocord, a European co-operative clinical-trial group for CBT of the European Blood and Marrow Transplantation (EBMT). Out of 171 patients, 45 with acute myeloblastic leukemia and 53 with acute lymphoblastic leukemia were included in a previously published study produced by Eurocord.

Design and Methods

Data collection and selection of patients

Eurocord is an international registry operating on behalf of the EBMT. Participation is open to both European and non-European centers conducting CBT. Eurocord works in...
close collaboration with Netcord banks. This retrospective analysis is based on data reported to the Eurocord Registry from European and non-European centers through a standardized questionnaire concerning the characteristics of patients, donors, diseases and grafts as well as transplant outcomes, reviewed by two physicians and checked for computerized errors to ensure data quality. The study included patients undergoing unrelated CBT between January 1996 and January 2003 who met the following criteria: (i) age ≥15 years old, (ii) diagnosis of a hematologic malignancy, (iii) conditioning with a myeloablative regimen, (iv) transplant with a single, non excess expanded cord blood unit and (v) not having received a previous allogeneic transplant. All patients gave informed consent for CBT according to the Declaration of Helsinki. The present study was approved by the Eurocord institutional review board.

**Patient, donor and transplant characteristics.**

The main patient, disease, transplant characteristics are reported in Table 1. A total of 171 patients reported from 63 centers in 13 countries (see Appendix) met the selection criteria. At the time of CBT, 82 patients (19%) with acute leukemia [ALL]; 3 with chronic myeloid leukemia [CML]; 7 with non-Hodgkin's lymphoma [NHL]; and 3 with myelodysplastic syndrome [MDS] had previously undergone an autologous transplant. Ninety-one patients (53%) were transplanted in an advanced phase of the disease (defined as AL in third or subsequent complete remission or refractory AL in [n=65], MDS [n=16], CML in blast crisis [n=6], or lymphoma in partial remission or in resistant relapse [n=9]). The median time from diagnosis to CBT was 13.4 months (range, 2.6-247), and median time from attainment of last complete remission to CBT in patients with AL transplanted in remission (n=75) was 129 days (range, 7-901). The median follow-up for survivors after transplant was 18.1 months (range, 1.5-70.7).

Donor-recipient compatibility for HLA-A and -B antigens was defined by serology, whereas the HLA-DRB1 match was defined at the antigen level by low-resolution DNA techniques and at the allelic level by high-resolution DNA techniques. HLA-DRB1 high resolution data were missing for only four (2.3%) donor-recipient pairs. The degree of matching was classified by the number (0, 1, 2 or 3) of HLA antigens or allelic disparities (Table 1). The HLA incompatibility involved antigens of class I in 65 pairs, class II antigens in 29 and both class I and class II antigens in 55 donor-recipient pairs. Most cord blood units (n=157, 92%) came from Netcord banks which followed the FACT-Netcord standards for collecting, freezing and storing cord blood units; the procedures for thawing and washing cryopreserved cord blood units followed the method described by Rubinstein et al.29

The median number of nucleated cells counted at the time of cord blood collection or freezing and infused are reported in Table 1. After thawing, there was a median loss of 24% (range: 4-52) of the nucleated cells CD34+ cell quantification at the time of infusion, calculated through non-standardized methods among centers and reported for only 124 cord blood units, was 1.0±0.1 cells (range: 0.02-15). Conditioning regimens differed according to centers, type of disease and disease status. An irradiation-based regimen with total body irradiation or total lymphoid irradiation was administered to 110 (64%) patients, while 61 patients were conditioned with combined chemotherapy only. In 129 (75%) cases the preparative regimen included an anti-thymocyte or anti-thymocyte globulin or a monoclonal anti-T-cell antibody. Most patients (n=117, 68%)

| Table 1. Patient, disease, donor and transplant-related characteristics. |
|---------------------------------|---------------|---------------|
| Characteristics | Number of patients (%) |
| (n-patients available) | or median (min-max) |
| Age, years (range) (n=171) | 29 (15-55) | |
| Male gender (n=171) | 64 (49%) | |
| Weight, kg (range) (n=169) | 62 (30-110) | |
| Diagnosis (n=171) | | |
| acute myeloid leukemia | 46 (27%) | |
| acute lymphoblastic leukemia | 33 (19%) | |
| secondary acute leukemia | 11 (6%) | |
| chronic leukemia | 8 (5%) | |
| lymphomas | 13 (8%) | |
| myelodyplasia | 16 (9%) | |
| Disease status at transplant (n=171) | | |
| early | 35 (21%) | |
| intermediate | 45 (26%) | |
| advanced | 51 (30%) | |
| Previous autologous transplant (n=169) | 32 (19%) | |
| Graft after 2000 | 106 (59%) | |
| Positive CMV serology (n=161) | 106 (66%) | |
| ABO compatibility (n=164) | 64(39%)/35(22%)/64(39%) | |
| Identical/minor/major disparities | | |
| HLA compatibility (n=169) | | |
| 6 out of 6 | 9 (5%) | |
| 5 out of 6 | 77 (46%) | |
| 4 out of 6 | 68 (41%) | |
| 3 out of 6 | 15 (9%) | |
| Number of cells at freezing | | |
| nucleated cells, 10^9/kg (n=169) | 2.7 (1.1-9.5) | |
| Number of infused cells | | |
| Nucleated cells, 10^9/kg (n=159) | 2.1 (0.8-7.3) | |
| CB34, 10^9/kg (n=124) | 1.0 (0.2-15.0) | |
| Conditioning (n=171) | | |
| Irradiation based | 110 (65%) | |
| TBI+Cy+Flu | 50 | |
| TBI+Cy+Flu+ATG | 40 | |
| TBI+Cy | 14 | |
| others (211) | 36 | |
| Busulfan based | 61 (36%) | |
| Bu+Cy+Fludarabine | 51 | |
| others | 10 | |
| Anti-T in the conditioning (ATG/ALG/MoAb) | 129 (78%) | |
| GVHD prophylaxis (n=171) | | |
| CSA alone | 11 (6%) | |
| CsA + Prednisone | 117 (69%) | |
| CsA+MTX+Prednisone | 18 (11%) | |
| FK+MX | 13 (8%) | |
| others | 12 (7%) | |
| Early hematopoietic growth factors (n=132) | 105 (69%) | |
received cyclosporine combined with steroids as prophylaxis against GVHD (Table 1).

Definitions of endpoints

Neutrophil engraftment. Myeloid engraftment was defined as a recovery of an absolute neutrophil count of at least 500/mm³ on 3 consecutive days. Platelet recovery was defined as the time needed to reach a sustained platelet count of at least 20,000/mm³ without transfusion support for 7 consecutive days. Absence of hematopoietic recovery at day 60, second transplantation or autologous hematopoietic reconstitution was considered as failure of engraftment.

Graft-versus-host disease. Starting on day 1, acute GVHD was scored according to the standard criteria² and counted only for grades II-IV. Patients surviving more than 100 days after transplant with sustained donor hematopoiesis were considered at risk for the development of chronic GVHD.²⁴

Relapse. Time to relapse was measured from the date of CBT to the date of disease recurrence as defined by morphological evidence of the neoplastic clone in either the bone marrow or in any extramedullary site. Patients who died in complete remission were censored at the date of death.

Transplant-related mortality. This was defined as all causes of death occurring at any time after CBT and not related to the underlying malignant disease.

Overall survival. Overall survival was measured as the time interval between the date of CBT and the date of death of any cause or the date of the last follow-up for survivors.

Disease-free survival. This was defined as the time interval between the date of CBT and the date of relapse or death in complete remission, whichever occurred first.

Statistical analysis

Variables related to the patients and donors (age, sex, sex match, cytomegalovirus serology, ABO compatibility, number of HLA disparities, disease- (acute and chronic leukemia versus other diseases, status at transplant, previous autologous transplant), and transplant (n. of nucleated cells at freezing and infused per kg, conditioning regimen, GVHD prevention) variables were analyzed for their potential prognostic value on each of the aforementioned endpoints. Univariate and multivariate proportional hazards
Table 2. Multivariate analyses of risk factors for the main outcomes after CBT for adults with hematopoietic malignancies.

<table>
<thead>
<tr>
<th>Outcomes* and unfavorable risk factors</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of nucleated cells collected ≥2.6×10^9/kg</td>
<td>2.02 (1.30-3.19)</td>
<td>0.009</td>
</tr>
<tr>
<td>Prolymphocytic hematopoietic growth factor</td>
<td>2.13 (1.31-3.47)</td>
<td>0.002</td>
</tr>
<tr>
<td>Relapse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diseases other than CML</td>
<td>10.98 (1.45-81.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Advanced status of the disease</td>
<td>1.47 (1.05-2.04)</td>
<td>0.04</td>
</tr>
<tr>
<td>Transplant-related mortality at 2 years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age ≥29 years</td>
<td>1.74 (1.08-2.82)</td>
<td>0.02</td>
</tr>
<tr>
<td>Female recipient</td>
<td>1.78 (1.13-2.73)</td>
<td>0.01</td>
</tr>
<tr>
<td>Survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female recipient</td>
<td>0.59 (0.40-0.91)</td>
<td>0.01</td>
</tr>
<tr>
<td>Major ABO incompatibility</td>
<td>0.63 (0.42-0.93)</td>
<td>0.05</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advanced status at the disease</td>
<td>0.59 (0.39-0.90)</td>
<td>0.015</td>
</tr>
<tr>
<td>Major ABO incompatibility</td>
<td>0.65 (0.43-0.98)</td>
<td>0.03</td>
</tr>
<tr>
<td>Female recipient</td>
<td>0.68 (0.46-0.96)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Risk factors for acute GVHD and platelets were not selected in the Cox model. CML, chronic myeloid leukemia; GVHD, graft-versus-host disease.

regression models were used to identify independent risk factors for death and disease-free survival by means of log-rank tests and Cox proportional hazards models.2× For assessment of factors predicting neutrophil and platelet recovery, acute GVHD (grade II-IV), chronic GVHD, relapse and transplant-related mortality, a similar methodology was used in a competing risks setting, death being treated as a competing event.2× Univariate and multivariate analyses were then performed using Gray’s test and the proportional subdistribution hazard regression model of Fine and Gray.2× A stepwise backward procedure was used to construct a set of independent predictors for each end-point. All predictors with a p-value below 0.10 were considered, and sequentially removed if the p-value in the multiple model was above 0.05. All tests were two-sided. The type 1 error rate was fixed at 0.05 for factors potentially associated with time-to-event outcomes. Since we found that the sex of the recipient was selected as a prognostic factor for transplant-related mortality and survival in a multivariate analysis without a clinical explanation, we also performed an analysis comparing recipient, donor, disease- and transplant-related factors according to recipient’s gender. All analyses were carried out using the cmprsk package (developed by Gray, June 2001) on Splus 2000 software and SPSS software.

Results

Neutrophil and platelet recovery

The cumulative incidence of neutrophil recovery at day 60 was 72±3% and the median time to reach an absolute neutrophil count of at least 500/mm³ was 28 days (range, 11-57) (Figure 1A). Of the 13 patients who did not recover neutrophil counts at day 60, four engrafted later between day 66 and 80 after transplant, five underwent a second transplant, three experienced autologous hematopoietic reconstitution, and one died in aplasia.

Twenty-six patients died early after transplant and before the median date of engraftment in the overall series (between days 4 and 28), thus the graft failure rate was 9% (18 out of 145 evaluable cases). In univariate analysis, a number of nucleated cells greater than 2.7×10^9/kg (81±5% vs 65±5%; p=0.02) at the time of freezing and early status of disease at transplant (89±5% for early vs 62±7% for intermediate vs 70±5% for advanced; p=0.05) were the two factors favorably affecting the probability of neutrophil recovery. In multivariate analysis, factors found to be significantly associated with neutrophil recovery were the number of nucleated cells at collection or freezing (relative risk [RR] 2.02, 95% confidence interval [95% CI]: 1.50 to 5.15; p=0.002) and the use of hematopoietic growth factors within one week after transplant (RR: 2.15, 95% CI: 1.81 to 3.47, p=0.002) (Table 2). The cumulative incidence of platelet recovery at day 180 was 46±4% and the median time to reach a platelet count of at least 20,000/mm³ was 84 days (range, 22-176) (Figure 1B). None of the analyzed factors was associated with the probability of platelet recovery in either univariate or multivariate analysis.

Acute and chronic graft versus host disease

Acute GVHD was counted as absent or grade 1 in 36 (21%) patients, grade II in 27 (16%), grade III in 15 (9%) and grade IV in 15 (7%). The cumulative incidence of acute GVHD at 100 days after CBT was 32±4% (Figure 1C). In univariate analysis, acute GVHD greater or equal to grade II was found to be more frequently associated with a diagnosis of CML than with AL or other malignant diseases (respectively, 50±8% vs 25±2% vs 36±7%, p=0.015). No other variables, including number and class distribution of the HLA disparities, were statistically associated with the incidence or severity of acute GVHD. In multivariate analysis, no factor was found to significantly influence the development or severity of acute GVHD. Chronic GVHD was observed in 34 of 92 patients at risk (limited in 18 and extensive in 16) with a cumulative incidence of chronic GVHD at 2 years of 36±10% (Figure 1D). In multivariate analysis, no factor showed a significant association with the occurrence of chronic GVHD.

Relapse

The cumulative incidence of relapse at 2 years was 22±4% for all patients (Figure 1E). It was 24±5% for patients with AL, 5±5 % for those with CML, and 31±12% for those with MDS. Three out of 13 patients transplanted for lymphomas relapsed. According to the disease status at transplant, the cumulative incidence of relapse at 2 years was 16±7% for patients transplanted in an early phase of the disease, 22±12% for those transplanted in intermediate status and 25±5% for those transplanted in advanced phase disease. In the Cox model, only two factors were significantly associated with an increased risk of relapse: diagnosis other than CML (RR 0.01, 95% CI: 0.01 to 0.69; p=0.02) and advanced disease status at the time of unrelated CBT (RR: 1.47, 95% CI: 1.00
to 2.1; p=0.04).

**Transplant-related mortality and causes of death**

The 2-year cumulative incidence of transplant-related death was 51±4% (Figure 1F). In univariate analysis, the incidence of transplant-related mortality was significantly higher in patients older than 29 years (59±5% vs 43±6%, p=0.02), in females (60±5% vs 45±6%; p=0.01), in patients transplanted in advanced phase (57±5% vs 45±6%; p=0.04) and in those with CML compared to those with AML or with other malignant diseases (76±5% vs 44±2% vs 49±7%, respectively; p=0.006). Furthermore, there was a trend for reduced transplant-related mortality in patients receiving cord-blood units containing a higher number of nucleated cells at collection or freezing (>2.6×10^7/kg), as compared to patients receiving a lower cell dose (45±6% versus 56±5%, p=0.06). In multivariate analysis, only two factors remained significantly associated with an increased risk of transplant-related death: age >29 years (RR 1.74, 95% CI: 1.05 to 2.89, p=0.02) and female gender (RR 1.78, 95% CI: 1.13 to 2.79; p=0.01) (Table 2).

**Causes of death.** Of 171 patients, 110 (64%) died and 61 are alive. Of the 110 deaths, 85 (77%) were due to transplant-related causes and most of them (n=68) occurred within 100 days after transplantation. The primary causes of death were disease relapse (n=25; 28%), infection (n=40; 36%), GVHD (n=12; 11%), acute respiratory distress syndrome or interstitial pneumonitis (n=7; 6%), hemorrhage (n=5; 5%) veno-occlusive disease (n=4; 4%); cardiac toxicity (n=3; 3%) multi-organ failure (n=8; 7%) and other causes (n=6; 5%).

**Overall survival and disease-free survival**

For all 171 patients, the 2-year probability of overall survival and disease-free survival was 38±4% and 27±4%, respectively. The probability of being alive without disease at 2 years was 41±3% and 34±10% for patients transplanted in an early and intermediate phase of disease, respectively (38±7 for early and intermediate phases of the disease) and 18±4% for patients transplanted in advanced phase (p=0.001, Figure 2). Table 3 shows the results of univariate analysis for disease-free survival. In multivariate analysis, advanced disease status at the time of CBT (RR 1.69; 95% CI: 1.1 to 2.5; p=0.015), female gender (RR 1.47; 95% CI: 1.01 to 2.17; p=0.05) and ABO major incompatibility (RR 1.55; 95% CI: 1.05 to 2.29; p=0.03) were identified as significant factors unfavorably affecting disease-free survival (Table 2). The relevance of patients’ gender in influencing the outcomes led us to analyze the frequency of all other variables according to the patients’ sex. No variables, including patient-, donor-, disease- and transplant-related factors or causes of death were found to be statistically different when analyzed according to patients’ gender (data not shown).

**Discussion**

This registry-based study confirms that cord blood represents a source of hematopoietic stem cells that can be successfully used for unrelated transplant not only in children but also in adults. Furthermore, the study provides information on outcomes and prognostic factors that should be taken into account when an unrelated cord blood graft is available for an adult patient. The reported experience on CBT in adults supports the feasibility of this strategy and very encouraging results have been obtained in single institutions.14,15 Moreover, two recent, registry-based studies showed similar outcomes for patients with acute leukemia transplanted with cord blood or bone marrow from unrelated donors.3,4 In the present analysis, the Eurocord Group extended the study to a large series of adult patients with different hematologic malignancies transplanted in 63 centers after 1997. In a previous study of 550 CBT recipients with malignant hematopoietic disorders, we showed that transplant-related mortality has been decreasing since 1998, due to better selection of cord blood units based on cell dose and number of HLA disparities.17 To avoid possible biases due to the study period we decided to include only those patients transplanted after 1998 in the present analysis.

In this study the time to hematopoietic engraftment was longer after CBT than that usually reported for HLA matched unrelated bone marrow transplants, in which the median number of nucleated cells infused is about ten times higher. However, the kinetics of engraftment in adults was similar to that reported for children undergoing CBT,18-20 in whom the cell dose infused is about 10-fold higher, due to their smaller size. Encouraging outcomes have been reported for adults with acute myeloblastic leukemia or MDS receiving CBT at a single center in Japan.21 The lower genetic diversity and the smaller size of the Japanese population may account in part for these results. The best results were achieved in patients grafted with ≥2×10^8 nucleated cells/kg of recipient’s body weight. This threshold of nucleated cord blood cells at infusion is recommended by Eurocord.23 Although the number of CD34+ cells and the number of colony-forming cells have been recognized to affect engraftment and patients’ outcomes significantly in children and adults, in this retrospective multicenter study, the low number of evaluable patients and the absence of standardized laboratory methods prevented us from assessing the possible impact of...
Table 3. Univariate analyses of disease-free survival (DFS) after CBT for adults with hematologic malignancies.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>2-year estimate of DFS (%)</th>
<th>P (log rank test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>27±4</td>
<td></td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>34±7</td>
<td></td>
</tr>
<tr>
<td>Acute myeloid leukemia</td>
<td>34±7</td>
<td></td>
</tr>
<tr>
<td>Secondary acute leukemia</td>
<td>22±13</td>
<td></td>
</tr>
<tr>
<td>Myelodysplastic syndrome</td>
<td>25±11</td>
<td></td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>19±7</td>
<td></td>
</tr>
<tr>
<td>Lymphomas*</td>
<td>13±10</td>
<td></td>
</tr>
<tr>
<td>Disease status*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early stage</td>
<td>41±9</td>
<td>0.001</td>
</tr>
<tr>
<td>Intermediate stage</td>
<td>34±10</td>
<td></td>
</tr>
<tr>
<td>Advanced stage</td>
<td>18±4</td>
<td></td>
</tr>
<tr>
<td>Time from diagnosis to UCBT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;13 months</td>
<td>31±5</td>
<td>0.38</td>
</tr>
<tr>
<td>≥13 months</td>
<td>24±6</td>
<td></td>
</tr>
<tr>
<td>No. of previous auto transplants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>27±5</td>
<td>0.30</td>
</tr>
<tr>
<td>≥1</td>
<td>23±8</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25 y</td>
<td>33±6</td>
<td>0.02</td>
</tr>
<tr>
<td>≥25 y</td>
<td>21±5</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 kg</td>
<td>28±7</td>
<td>0.99</td>
</tr>
<tr>
<td>≥60 kg</td>
<td>24±6</td>
<td></td>
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<tr>
<td>CMV serology</td>
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<td></td>
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<tr>
<td>Negative</td>
<td>35±7</td>
<td>0.17</td>
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<tr>
<td>Positive</td>
<td>23±5</td>
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</tr>
<tr>
<td>Recipient's gender</td>
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<td></td>
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<tr>
<td>Male</td>
<td>29±6</td>
<td>0.05</td>
</tr>
<tr>
<td>Female</td>
<td>25±5</td>
<td></td>
</tr>
<tr>
<td>ABO compatibility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matched</td>
<td>32±5</td>
<td>0.07</td>
</tr>
<tr>
<td>Minor mismatch</td>
<td>19±6</td>
<td></td>
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<tr>
<td>Major mismatch</td>
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<tr>
<td>HLA disparities (DRBEI high resolution)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 out of 6</td>
<td>44±17</td>
<td>0.18</td>
</tr>
<tr>
<td>5 out of 6</td>
<td>20±5</td>
<td></td>
</tr>
<tr>
<td>4 out of 6</td>
<td>30±7</td>
<td></td>
</tr>
<tr>
<td>3 out of 6</td>
<td>40±16</td>
<td></td>
</tr>
<tr>
<td>NC at freezing/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.7x10^10</td>
<td>28±5</td>
<td>0.32</td>
</tr>
<tr>
<td>≥2.7x10^10</td>
<td>20±6</td>
<td></td>
</tr>
<tr>
<td>NC infused/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.1x10^10</td>
<td>25±5</td>
<td>0.25</td>
</tr>
<tr>
<td>≥2.1x10^10</td>
<td>29±6</td>
<td></td>
</tr>
<tr>
<td>CD34 infused/kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1.0x10^10</td>
<td>29±6</td>
<td>0.46</td>
</tr>
<tr>
<td>≥1.0x10^10</td>
<td>35±6</td>
<td></td>
</tr>
<tr>
<td>Growth factor day 0-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>16±8</td>
<td>0.51</td>
</tr>
<tr>
<td>Yes</td>
<td>33±5</td>
<td></td>
</tr>
<tr>
<td>Irradiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>29±6</td>
<td>0.31</td>
</tr>
<tr>
<td>Yes</td>
<td>26±5</td>
<td></td>
</tr>
<tr>
<td>Year of UCBT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2000</td>
<td>23±5</td>
<td>0.44</td>
</tr>
<tr>
<td>≥ 2000</td>
<td>32±5</td>
<td></td>
</tr>
</tbody>
</table>

SD: standard deviation; CMV: cytomegalovirus; UCBT: unrelated cord blood transplant. *Non-Hodgkin's lymphoma = 12; Hodgkin's lymphoma = 1. **BMTR classification (see Table 1).
Unrelated cord blood transplants in adults

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References


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Ethical and legal aspects of placental/cord blood banking and transplant

E Gluckman* for Eurocord Netcord Organisation

*Project leader of Eurocord; Head, Bone Marrow Transplant Unit; Hôpital Saint Louis, Paris, France

Introduction

As more than 1000 unrelated cord blood transplants (CBT) have been performed worldwide, the number of cord blood banks (CBB) has been increasing with more than 25,000 units collected and available for unrelated donor haematopoietic stem cell transplant searches. The main advantages of CBT are the large donor pool, the low incidence of viral infection at birth, the low incidence of graft versus host disease (GVHD) due to the immune immaturity of the new-born and the increased speed of search as the units are readily available, having been previously tested and cryopreserved. Since placental/umbilical cord blood banking for unrelated transplantation is a relatively new field, it is very important to set up minimum standards and to reach an international agreement on aspects essential for the safety of the donor and the mother and the opportunity to offer the best possible chance for the recipient. For this purpose, Netcord was founded in 1998. It currently includes experienced placental/cord blood banks (Barcelona Spain, Denver USA, Duesseldorf Germany, Leiden Netherlands, London UK, Milan Italy, New York, NY, USA, Paris France, St Louis USA and Tokyo Japan). The joint inventory available on a single research file currently contains 28,650 validated units; 1017 have been transplanted to paediatric and 272 to adult patients. The Board of directors of Netcord has developed a detailed set of standards for cord blood banking. Eurocord is closely linked to Netcord: it is an international Registry of CBT which has already collected and analysed more than 500 CBT and performs analysis of the clinical results and implements clinical studies in order to evaluate the efficacy of CBT.

Since the first cord blood transplant was performed in 1998, several ethical issues have been raised concerning the status of cord blood haematopoietic stem cells, the issue of consent and ownership and the problems of privacy and commercialism.1-9

Status of umbilical cord blood cells

In contrast with other sources of haematopoietic stem cells which are collected for allogeneic transplant on a living consenting donor, cord blood is considered as a discarded product at the completion of pregnancy. According to different laws, cord blood cells can be considered as a blood product, a tissue or an organ transplant. Because of this difference, some might argue that the collection of cord blood cells should be governed by rules which do not require that an individual gives permission for the use of discarded tissues. In fact, because of the necessity to collect information on the medical status of the mother and the infant, to perform tests for infectious or genetic diseases and because the cells are used for a medical purposes on an anonymous patient, all agreed upon the necessity of requiring an informed consent before the delivery which is accepted and signed by the mother and if possible by both parents. Usually, the rules are those observed for unrelated bone marrow donors which stipulate that the gift is anonymous and that there will not be any financial compensation.

Consent, safety and privacy

According to Netcord standards, the following procedure must be followed for consent and ensuring the safety of the products delivered to the patients:

- Information about the program should be made available to the mother during the antenatal period.
- Informed written consent from the mother is required before, or less than 48 h after, collection.

The mother’s informed written consent must authorise the following:

- Storage of her CB for use by any patient who requires it.
- The disposal by the bank of units not meeting the criteria for banking after removing the link to the individual mother.
• The sample may be used for testing, research or for other valid purposes.
• The collection of a sample of her blood for testing for infectious diseases such as HIV and reporting back to her the results of this testing and that of the baby's blood through a physician of the mother's choice. This sample shall be obtained no more than 3 days preceding the delivery or 30 days following it. Similar tests shall be done on an aliquot of the baby donor's CB serum or plasma.
• The collection of data regarding her personal and family histories with focus on the existence of risk factors or a possible exposure to infectious and genetic diseases of potential importance in the context of stem cell allotransplantation.
• A review of the mother's hospital chart and that of the baby for further antecedents suggestive of risk factors and to record the diagnoses made prior to discharge.
• The transfer of data relating to the cord blood donation anonymously to transplant centers or registries world-wide either on paper or electronically.
• The storage of appropriate samples including cells and DNA for future testing.
• The collection of cord blood during the 3rd stage of labour when that is the strategy chosen for collection.
• The collection of a swab sample of baby saliva for CMV culture if this is the program method for neonatal screening.

Minimum tests for infectious diseases screening include bacteriological screening for aerobes and anaerobes in each unit collected before freezing. The following tests are mandatory on the mother: HbsAg, anti HCV, anti HIV 1/2, anti CMV-IGM, syphilis. In addition, national recommendations to test for HIV PCR or for antibodies against infectious diseases of regional relevance such as anti CMV IgG, HTLV 1/2, toxoplasma, EBV and parvovirus B 19 will be performed under the respective SOP of Netcord banks. The same testing shall be performed on the CB unit itself prior to release.

Information to the patients (from Eurocord protocol)

Why use cord blood?

It was demonstrated, several years ago, that the blood present in the placenta at birth is enriched in haematopoietic stem cells. The number of haematopoietic stem cells present in cord placental blood is equivalent to the amount given in a bone marrow transplant.

These cells also have the advantage of being less immunologically reactive; they are expected to give less graft versus host disease than adult cells. For this reason a complete HLA identity is not an absolute prerequisite for indication of a cord blood transplant.

How is cord blood collected?

After birth, the cord is clamped and resected. Blood is immediately collected in the umbilical vein. After collection, cord blood is transported in a bank where it is frozen in liquid nitrogen after a sample has been taken for testing.

What are the tests performed?

The mother signs an informed consent for the donation of cord blood cells. It is explained that cord blood donation is anonymous and that the mother cannot receive any payment for her donation. If a haematopoietic stem cell transplant is needed in her family, she cannot claim any ownership of her cord blood. A questionnaire is given to enquire about family history in order to detect any criteria for exclusion of the donation including genetic or infectious diseases. In addition, the mother is tested for infectious diseases before and after donation. After collection, the cord blood is tested for HLA typing, number of cells and infection. The results of HLA typing are sent to a Registry for utilisation. When cord blood is selected for a patient, HLA typing and infectious disease markers are repeated on a sample which is kept separately at the collection for further testing. All efforts are made to avoid any risk of transmission of genetic or infectious disease transmission with the transplant, in agreement with current regulations.

Why a cord blood transplant?

In your case, your physician thinks that an haematopoietic stem cell transplant is the best treatment of your disease. An HLA compatible donor was not found in your family nor in international registries of unrelated bone marrow donors. For this reason, we have selected a cord blood as closely matched as possible.

Modalities of transplantation

Utilisation of cord blood cells does not modify the usual technique of haematopoietic stem cell transplant which involves conditioning, graft versus host disease (GVHD) and infectious disease prevention. Placental blood is thawed in a water bath and infused immediately through a central venous catheter.

The main risks are rejection, GVHD and infectious complications. On long-term follow-up, other complications can occur, including relapse or chronic GVHD. These complications can also occur after a standard bone marrow transplantation.

This is a research protocol. More than 1200 patients have been transplanted with familial or unrelated cord blood world-wide; the first cord blood transplant was performed in 1988. Preliminary results are very
encouraging but more cases and more follow-up are needed for an analysis of long-term results.

Information concerning your case will be sent to a central Registry for analysis of the results. Your name will not appear and the information will be kept confidential.

Commercialism

Eurocord has been very active in opposing a patent on cord blood cells. This patent covered "haematopoietic stem and progenitor cells of neonatal and fetal blood and the therapeutic uses of such cells upon thawing." The patent has been successfully opposed by Eurocord because of lack of novelty, lack of an inventive step and lack of enabling disclosure (decision of the European Patent Office in Munich, 8 June 1999). The patent office was convinced by several papers describing similar techniques that predated the patent. The patent has already been rejected in the USA and in Japan. This result has been obtained on legal grounds but it is also an ethical victory as it overturns a patent on human tissues.

Reports of successful CBT captured the interest of parents who expressed an interest in preserving a cord blood unit (CBU) in the event that either the newborn or a sibling might one day require a transplant. Occasionally, parents even plan pregnancy as a means to develop access to a transplant and intend to use the CBU almost immediately. Otherwise, since transplant is used to treat mostly rare diseases, the enthusiasm to maintain storage of a self or family-directed CBU is often diminished over time due to cost considerations.

Mostly commercially motivated CBT banks have sought participation from expectant mothers who agree to pay for storage of a CBU from their newborn infant. The infrequent utilisation of a related CBU does minimise its utility. The probability that the cord blood will be of use in a family with no history of blood disease approaches zero, moreover, one's own stem cells may be less effective in treatment than those of an unrelated donor. In addition, it has been shown recently that leukemic cells were found at birth in children diagnosed with acute leukemia for periods as long as 5–10 years. This demonstrates the latency of the disease and indicates that some leukemias can be acquired in utero. Furthermore, mounting costs have cast a doubt on the viability of such banks. There is an increasing call from the public sector to encourage volunteer donation of CBU to banks responding to the need of the unrelated general public. A central policy issue is whether obstetricians should encourage patients to choose the storage of placental blood for their own use (by for-profit companies) or to donate placental blood for the others (through not-for-profit organisations).

Economic issues are a very important consideration in the development of cord blood banks, the total cost of a single unit delivered to a patient has been estimated at 15 000 euros. This has to be considered carefully when planning the development of cord blood banks for allogeneic use. The main advantage of cord blood transplant is the decrease of GVHD, which opens the way to performing mismatched transplants while the results of unrelated bone marrow transplants are strongly dependent on HLA class I and II identity. This factor explains why, with a total of more than 5 million bone marrow donors available world-wide, the probability of finding a perfect match is only 50% in the white caucasian population while the probability of finding a 1 or 2 antigen mismatched cord blood donor in a bank of 25 000 units is close to 100%, providing that the number of cells be superior to 2 x 10^9/kg patient body weight.

Clearly, cord blood transplant is still an experimental procedure which requires co-operative protocols, carefully designed prospective studies, evaluation of the results and comparison with other therapies. Eurocord and Netcord are working together to provide the best unit to the patient in order to deliver the best possible care to all patients who need a transplant.

References


Will stem cells in cord blood, amniotic fluid, bone marrow and peripheral blood soon be unnecessary in transplantation?

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There are now various sources of stem cells. Those derived from blastocysts, named embryo stem (ES) cells, have attracted most attention and are highly multipotent. Human cord blood became widely used as a source of stem cells with differing properties to ES cells and their therapeutic application has grown steadily as they are stored in increasing numbers of stem cell banks. Other sources of human stem cells are derived from peripheral blood and amniotic fluid. They may arise from a common origin in epiblast. This review stresses the use of cord blood stem cells, but describes new approaches which may supersede the use of most stem cells. The advantages and disadvantages of these various classes are described in relation to potential methods involving gene conversion to change somatic cells to ES cells.

PMID: 17359598 [PubMed - indexed for MEDLINE]
Is prenatal HLA typing of uncultured amniocytes before the collection of related allogenic cord blood helpful?

Abstract The collection of related allogenic cord blood is gaining increasing importance in families with one child affected by haematopoietic disease. Within a family, there is only a 25% chance of a full HLA match between siblings. 50% of all collected cord blood samples cannot be used because of poor quality. Because of this, the determination of HLA type is useful for planning the collection of related allogenic cord blood transplants. We studied whether HLA typing is possible during late pregnancy if amniocentesis has not been performed during the first trimester. HLA-A, -B and -DRB loci were detected in amniotic fluid, as well as in corresponding cord blood and maternal blood using PCR-SSP. For the first time, HLA typing was performed from uncultured amniocytes. Unambiguous results were obtained from all samples. Fetal HLA-genotype in amniotic fluid was confirmed by typing results from corresponding cord blood. HLA typing of uncultured amniocytes during late pregnancy is a reliable and fast method. For the first time, prenatal HLA typing by amniocentesis after week 38 of gestation is possible in less than 8 h and without fetal risk.

Keywords Stem cell transplantation · Prenatal HLA typing · Uncultured amniocytes

Introduction

Molecular genetic techniques for the examination of amniocytes from amniotic fluid are not only an essential tool in prenatal diagnostic for chromosomal aberrations but can also be used for prenatal determination of the HLA-genotype.

Allogenic transplantation of haematopoietic stem cells from umbilical cord blood of HLA identical siblings (related allogenic cord blood) is a therapeutic concept of increasing importance. In families with one child affected by haematopoietic disease, prenatal diagnosis of the HLA-genotype has moved into the centre of interest [1, 2].

In 25% of cases, the donating sibling has a complete HLA match with the affected sibling, whereas, in 50%, the use of an HLA-haploidentical CB has to be discussed critically with the transplant centre. In 25%, transplantations cannot be performed because of HLA-incompatibility.

If HLA typing has not been done until the day of delivery, there is the danger that a potentially curative transplant cannot be used because of inadequate precautions.

In the present study, we examined, for the first time, the possibility of fetal HLA typing from uncultured amniocytes during the last trimester and discuss clinical consequences.

Materials and methods

Amniotic fluid samples were collected during planned caesarean sections after week 38 of gestation. It was possible to collect nine portions of 22–25 ml, while one portion contained only 4 ml of amniotic fluid. Amniotic fluid samples were examined together with corresponding umbilical cord blood and maternal blood. Among these ten specimens were two twin pregnancies with different types of placenta. One of these was monochorionic, the other one dichorionic.

Amniotic fluid samples were stabilised by EDTA containing buffer solution. One sample from a singleton pregnancy showed a slight contamination by maternal blood. Amniotic fluid was centrifuged at 1000 x g for 10 min. The cell pellet was resuspended in 200 μl PBS (phosphate buffered saline; Gibco BRL). The QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) was used to isolate DNA from 200 μl amniocyte suspension, as well as from corresponding 200 μl maternal blood and 200 μl cord blood samples. DNA was recovered from the spin columns by using 220 μl 1 M TRIS-HCL pH 8.3. DNA quality was evaluated by estimating the mean molecular weight and the presence of degradation products. PCR-SSP – (polymerase chain reaction – sequence specific primers) low resolution typing of HLA-A, -B and -DRB loci was per-

Prenatal HLA typing of uncultured amniocytes prior to the collection of related allogeneic cord blood.

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Institute of Transfusion Medicine and Clinical Immunology, Red Cross Blood Service of Baden-Württemberg, Mannheim, Germany.

DNA samples isolated from corresponding uncultured amniotic fluid, cord blood and maternal blood (n=5) were subjected to low resolution typing of the HLA-A, -B and -DRB loci by the polymerase chain reaction using sequence-specific primers (PCR-SSP). Furthermore, the effect of ethylene diamine tetraacetate disodium salt (EDTA) on the quality of genomic DNA isolated from amniotic fluid samples after long-term storage was evaluated. Unambiguous results of HLA typing could be achieved from all amniotic fluid samples stabilized with EDTA. PCR-SSP typing failed in DNA samples from amniotic fluid without the addition of EDTA. In all cases the fetal HLA type could be confirmed by the result from the corresponding cord blood typing. Contamination with maternal DNA led to additional weak PCR-SSP bands in one case, but data interpretation was still unambiguous. Reliable fetal HLA typing can be achieved directly from amniotic fluid and culturing of amniocytes is not required.

PMID: 11696225 [PubMed - indexed for MEDLINE]
Liver stem cells: a scientific and clinical perspective.

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The promise of liver stem cells lies in their potential to provide a continual and readily available source of liver cells that can be used for gene therapy, cellular transplant, bioartificial liver-assisted devices, drug toxicology testing and use as an in vitro model to understand the developmental biology of the liver. Both the rodent and human embryonic stem cell, bone marrow hematopoietic stem cell, mesenchymal stem cell, umbilical cord blood cell, fetal liver progenitor cell, adult liver progenitor cell as well as the mature hepatocyte have been reported to be capable of self-renewal, giving rise to daughter hepatocytes both in vivo and in vitro. These cells can repopulate livers in animal models of liver injury and seemingly improve liver function. However, significant challenges still exist before these cells can be used in humans. These include lack of consensus in immunophenotype of liver progenitor cells, uncertainty of the physiological role of reported candidate stem/progenitor cell, practicality in obtaining sufficient quantity of cells for clinical use and concerns over ethics, long-term efficacy and safety. Current molecular techniques of stem cell identification are confounded by cell fusion, horizontal gene transfer, incomplete differentiation and fetal microchimerism. Reports of stem cell transplantation and phase 1 trials of bone marrow transplantation in humans for liver diseases are exciting but require more robust verification. We review the evidence for various candidate stem cells, human clinical trials reported to date and highlight the challenges facing clinicians in their quest to use liver stem cells to save lives.

PMID: 18410603 [PubMed – indexed for MEDLINE]
1. Stem Cells Dev. 2007 Apr;16(2):281-96.

Phenotypic and functional analysis of human fetal liver hematopoietic stem cells in culture.

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Steady-state hematopoiesis and hematopoietic transplantation rely on the unique potential of stem cells to undergo both self-renewal and multilineage differentiation. Fetal liver (FL) represents a promising alternative source of hematopoietic stem cells (HSCs), but limited by the total cell number obtained in a typical harvest. We reported that human FL nonobese diabetic/severe combined immunodeficient (NOD/SCID) repopulating cells (SRCs) could be expanded under simple stroma-free culture conditions. Here, we sought to further characterize FL HSC/SRCs phenotypically and functionally before and following culture. Unexpanded or cultured FL cell suspensions were separated into various subpopulations. These were tested for long-term culture potential and for in vivo repopulating function following transplantation into NOD/SCID mice. We found that upon culture of human FL cells, a tight association between classical stem cell phenotypes, such as CD34(+) /CD38(-) and/or side population, and NOD/SCID repopulating function was lost, as observed with other sources. Although SRC activity before and following culture consistently correlated with the presence of a CD34(+) cell population, we provide evidence that, contrary to umbilical cord blood and adult sources, stem cells present in both CD34(+) and CD34(-) FL populations can sustain long-term hematopoietic cultures. Furthermore, upon additional culture, CD34-depleted cell suspensions, devoid of SRCs, regenerated a population of CD34(+) cells possessing SRC function. Our studies suggest that compared to neonatal and adult sources, the phenotypical characteristics of putative human FL HSCs may be less strictly defined, and reinforce the accumulated evidence that human FL represents a unique, valuable alternative and highly proliferative source of HSCs for clinical applications.

PMID: 17521239 [PubMed - indexed for MEDLINE]
SPECIAL REPORT

The EBMT activity survey 2007 with focus on allogeneic HSCT for AML and novel cellular therapies

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The 2007 report describes the current status of HSCT activity in Europe, highlights the increasing role of allogeneic HSCT in treatment of AML and gives the first quantitative information on novel cellular therapies. In 2007, there were 25563 first HSCTs, 10072 allogeneic (39%), 15491 autologous (61%) and 3606 additional transplants reported from 613 centers in 42 countries. The main indications were leukemias (8061 (32%; 89% allogeneic)); lymphomas (14627 (57%; 89% autologous)); solid tumors (1488 (6%; 96% autologous)) and nonmalignant disorders (1302 (5%; 91% allogeneic)). Peripheral blood was the main source of stem cells for autologous HSCT (98%) and the predominant source for allogeneic HSCT (71%). Among allogeneic HSCTs, the number of unrelated donor grafts equaled the number of HLA-identical sibling donor grafts for the first time (47% each). AML was the most frequent indication for allogeneic HSCT (32% of all allogeneic HSCTs), with an increase of 247 (8%). Information on novel cellular therapies was collected for the first time; there were 212 mesenchymal SCTs and 212 HSCTs for nonhematopoietic use. The indications for the latter were cardiovascular disorders (97; 46%), neurological disorders (94; 44%) and tissue repair (21; 10%). These data illustrate the expanding role of cellular therapies.

Bone Marrow Transplantation (2009) 43, 275–291; doi:10.1038/bmt.2009.7; published online 26 January 2009

Keywords: hematopoietic SCT; Europe; transplant rates; acute myeloid leukemia; mesenchymal stem cells; novel cellular therapies

Introduction

The annual European Group for Blood and Bone Marrow Transplantation (EBMT) activity report has become an established instrument to describe the current status of HSCT in Europe to observe trends and to monitor changes in technology use.1 2 It serves as a basis for decision making at the individual patient level as well as for health care agencies in planning and providing the infrastructure for this complex medical technology. In addition to the general description of the number of transplants by indication, donor type and stem cell source, the report has focused each year on specific aspects. The increasing use of cord blood as a stem cell source, the change from BM to peripheral blood or the utilization and integration of unrelated donor transplants were the key topics in the past.3 4 In the 2007 report, information on the use of novel cellular therapies was integrated for the first time. The numbers of mesenchymal SCTs and the numbers of HSCT for nonhematopoietic indications were requested.5 6 The most striking observation in the last year was the increase of allogeneic HSCTs for the treatment of AML and myelodysplastic syndromes (MDS). More detailed information is provided on the pattern of use for this indication.

Patients and methods

Data collection and validation

All participating teams were requested to report their data for 2007 by indication, stem cell source and donor type as listed in Table 1. Data were validated by three independent systems: through confirmation by the reporting team that received a computer printout of the entered data, by selective comparison with MED-A data sets in the EBMT ProMSE data system and by cross-checking with the National Registries. Onsite visits of selected teams were part of the quality control program (www.jacie.org).

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Teams
A total of 628 teams in 45 countries (38 European and 7 affiliated countries) were contacted for the 2007 report, of which 613 reported their numbers. This corresponds to a 98% return rate of active teams and includes 509 active EBMRT member teams reporting to the survey. There were 15 teams known to have been performing HSCTs in 2007 that chose not to reply or failed to reply. The teams that were contacted are listed in the Appendix in alphabetical order according to country, city and EBMT center code. According to the information received, there were no bone or marrow transplants performed in Albania, Andorra, Armenia, Georgia, Liechtenstein, Malta, Moldavia, Monaco, Montenegro, San Marino and the Vatican in 2007. The non-European countries participating in the EBMT survey include Algeria, Iran, Israel, Lebanon, Saudi Arabia, South Africa and Tunisia. Their data are included in some of the analyses.

Definitions
Transplant numbers. The EBMT survey focused, as in previous years, on the number of patients treated for the first time with HSCT. 1 Information on additional transplants, for instance, a second, third or fourth HSCT in a patient with a previous HSCT was collected by disease category only for those patients with a planned double autologous after autologous transplants; for all other situations, this information was collected generically only. The following definitions were used: 'retransplants' (autologous or allogeneic) were defined as an unplanned HSCT for rejection or relapse after a previous HSCT; multiple transplants were defined as being part of a planned double or triple autologous or allogeneic transplant protocol. Information on stem cell source was collected as BM, peripheral blood or cord blood. Any transplants with a combination of stem cell source that included cord blood were reported as cord blood HSCTs. BM and peripheral blood combinations were reported as peripheral blood HSCTs. Information on reduced-intensity conditioning (RIC) was collected as a total for each team only and not for individual transplants. Definitions for RIC HSCT followed the recently published definitions.11

Transplant rates. Transplant rates were computed as the number of HSCTs per 10 million inhabitants as defined earlier.12 Transplant rates refer to the number of transplants in a given country compared with its own population. The survey cannot make adjustments for patients who cross borders and receive their HSCT in a foreign country. Population data were obtained from the US Census office (http://www.census.gov).

Economic factors. Economic factors considered in the analysis followed previously defined rules.12 Countries were categorized by their Gross National Income (GNI) per capita according to the World Bank definitions into high income (Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Ireland, Italy, the Netherlands, Norway, Portugal, Slovenia, Spain, Sweden, Switzerland and the United Kingdom), middle income (Bulgaria, Croatia, Hungary, Latvia, Lithuania, Poland, Romania, Russia, Serbia, Slovakia and Turkey) and low income countries (Azerbaijan, Belarus, Bosnia and Herzegovina, Macedonia and Ukraine). The latter category refers to the World Bank definition of 'lower middle income' (http://www.worldbank.org). Furthermore, the category of high income was subdivided into a very high-income group, consisting of those countries with a GNI/capita of >40 000 per capita (Denmark, Finland, Ireland, the Netherlands, Norway, Sweden, Switzerland and the United Kingdom).

The non-European countries that traditionally participate in the EBMT activity survey (Algeria, Iran, Israel, Lebanon, Saudi Arabia, South Africa and Tunisia) are included in the overall data presentation. They were not included in the analysis on economic factors. The same applies to Iceland and Luxembourg because of some missing data over the time span.

Statistical analysis. The relation of the macroeconomic factors (GNI/capita) with transplant rates was estimated by ordinary least squares by multiple regressions to measure the coefficient of determination (r²) or explanatory content.

Results
Participating teams
Of the 613 teams reporting HSCTs in 2007, 374 (61%) performed both autologous and autologous transplants; 225 (37%) restricted their activity to autologous transplants, 5 teams (1%) to autologous transplants only and 9 teams (1%) reported having performed no transplants in 2007.
A total of 216 teams (35%) reported fewer than 20 first HSCTs in 2007, 223 teams (37%) between 20 and 50 HSCTs, 121 teams (20%) between 51 and 100 HSCTs and 53 teams (8%) >100 HSCTs.
A total of 142 teams reported at least one cord blood HSCT in 2007 with 33 teams reporting >5.

Numbers of HSCT in 2007
First transplants in 2007. A total of 25 563 first transplants, 10 072 (39%) allogeneic and 15 491 (61%) autologous, were carried out in 2007 (Table 1). Overall, this corresponds to a slight increase in the numbers of HSCT compared with 2006 when there were 25 050 first transplants. The numbers of allogeneic HSCT increased by 4% from 9661 in 2006 to 10 072 in 2007, whereas the numbers of autologous HSCT remained similar at 15 389 in 2006 and 15 491 in 2007.

Additional transplants in 2007. There were 1662 retransplants (810 allogeneic/852 autologous) and 1944 additional planned multiple transplants (71 allogeneic/1873 autologous). Thus, there were a total of 29 169 HSCT procedures, 10 953 allogeneic (38%) and 18 216 autologous (62%), performed in 2007. This corresponds to an overall increase of 105 retransplants (38 allogeneic and 67 autologous) or 7% compared with 2006. A total of 522 transplants were reported as being part of a planned double autologous-
allogeneic HSCT. This corresponds to a decrease of 5% when compared with 2006, where a total of 531 planned double autologous-allogeneic HSCTs were reported. The main indications for the planned double transplant programs were, as in the previous year, multiple myeloma, non-Hodgkin's lymphoma and Hodgkin's disease. The evolution over time in the number of participating teams, numbers of allogeneic and autologous HSCTs is depicted in Figure 1.

Transplant rates in 2007. There were marked differences in transplant rates between European countries and countries affiliated with EBMT as presented in Figure 2. These differences relate to all transplants (Figure 2a) and to autologous HSCT (data not shown). The differences between Eastern and Western European countries have been reported earlier. It is interesting to note that countries with similar total transplant rates had similar transplant rates for allogeneic HSCT as well as for autologous HSCT.

Disease indications in 2007
The indications for HSCT in 2007 are listed in detail in Table 1. The main indications were 'lymphoproliferative disorders' with 14,627 patients (57%), 1,646 patients with allogeneic HSCTs (11%), 12,981 with autologous HSCTs.
(89%); 'leukemias' with 8061 patients (32%), 7153 patients with allogeneic HSCTs (89%), 908 with autologous (11%) HSCTs; 'solid tumors' with 1488 patients (6%), 63 with allogeneic HSCTs (4%), 1425 with autologous HSCTs (96%); and 'nongenetic disorders' with 1302 patients (5%), 1141 with allogeneic HSCTs (91%) and 161 with autologous HSCTs (12%). The latter, autologous HSCT for nongenetic disorders, predominantly includes patients (150) with autoimmune disorders. An additional 85 patients (0.5%), 69 with allogeneic HSCTs and 16 with autologous HSCTs, were reported as 'other indications'.

Stem cell source in 2007

Of the 15491 autologous first transplants, 256 (2%) were BM derived, 15234 (98%) were from peripheral blood stem cells or from combined BM and peripheral blood stem cells and one was from autologous cord blood cells (Table 1). Of the 10072 allogeneic first transplants, 23% were BM, 71% were peripheral blood and 6% were cord blood transplants. This corresponds to a stable proportion of peripheral blood as stem cell source compared with the 70% in 2006. The proportion of peripheral blood as stem cell source varied depending on donor type. It was 73% for HLA-identical sibling donor transplants, 68% for unrelated donors, 74% for HSCT from other family members and 73% for twin donors. Within allogeneic HSCT, the only disease indications with more BM than peripheral blood donors as stem cell source were BM failure syndromes (51% bone marrow) and congenital disorders (59% BM). The proportion of main indications varied as well within the three stem cell sources. Nonmalignant disease represented about a quarter of all indications for BM and cord blood but only a small fraction among the peripheral blood transplants.

Donor type in 2007

For the 10072 allogeneic first transplants, HLA-identical siblings were used as donors for 4716 (47%) of the recipients, other family members for 552 (5%) of the recipients, a syngeneic twin for 52 (1%) of the recipients and an unrelated volunteer donor for 4752 (47%) of the recipients. For the first time since the introduction of the EBMT activity survey, the proportion of unrelated donors was higher than the number of HLA-identical sibling donors.

Use of RIC in 2007

The numbers of RIC HSCT continued to increase from 3530 in 2006 to 3914 in 2007 at the same rate as allogeneic HSCT. RIC was used for 36% of all allogeneic HSCT. This information is collected in a generic way only; no information on disease distribution is possible within the activity survey.

Focus of the 2007 survey

AML. AML was the most frequent indication for an allogeneic HSCT in 2007. The 3269 allogeneic HSCTs for AML correspond to 32% of all allogeneic HSCTs. Together with the 1052 HSCTs for MDS, AML and MDS (4321 allogeneic HSCTs) correspond to 43%. There were more HSCTs for AML in the first CR (58%) compared with later stages of the disease. HSCT for AML in the first CR increased from 1354 in 2004 to 1903 in 2007. This is the highest increase for any indication over the recent years (Figure 3). Allogeneic HSCTs for all patients with AML increased from 2404 in 2004 to 3269 in 2007. In contrast, the numbers of autologous HSCT for AML in the first CR declined from 874 in 2004 to 577 in 2007.

Transplant rates for allogeneic HSCTs in AML and MDS differed significantly between participating countries (Table 2) and ranged from less than 5 per 10 million (several countries) to 135 per 10 million inhabitants in Belgium. Countries with high transplant rates for AML also showed high transplant rates for MDS, but transplant rates for AML did not necessarily parallel transplant rates in general (Figures 2b, c and d). It is interesting to note that transplant rates for AML in the first CR were very homogeneous with a coefficient of variation in high-income countries of 39, indicating a consensus among the participating countries. The transplant rates in high-income countries for MDS varied more with a coefficient of variation of 68; for autologous HSCT for AML (Figure 2d), the variation was even larger with a coefficient of variation of 119.

Similarly, the coefficient of determination for GNI/capita and transplant rates differed significantly between allogeneic and autologous HSCTs. It was high for AML allogeneic HSCT with an explanatory content of $r^2=64.27\%$, MDS allogeneic HSCT with $r^2=66.21\%$ or AML first CR allogeneic HSCT with $r^2=49.54\%$. It was low for autologous HSCT with $r^2=9.91\%$ for AML autologous HSCT, $r^2=9.21\%$ for MDS and $r^2=8.47\%$ for AML first CR only.

Novel cellular therapies. Table 3 summarizes the experience in Europe with novel cellular therapies. There were a total of 212 mesenchymal SCTs performed by 46 teams in 15 countries. The indications for these transplants are unknown. There were a total of 212 HSCTs for non-hematopoietic use. This includes 97 HSCTs for cardiovascular disorders, 94 HSCTs for neurological disorders and 21 HSCTs for tissue repair.

There were 1898 patients reported as having received donor lymphocyte infusions in 2007 (Table 3). This corresponds to about two-thirds of the number of reported patients with RIC HSCT. No information on the disease indication of those patients with donor lymphocyte infusion is available from the activity survey.
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**Table 2 Numbers and transplant rates of allogeneic HCTs for AML and MDS in Europe in 2007**

**Demographics**

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**Table 2 Numbers and transplant rates of allogeneic HCTs for AML and MDS in Europe in 2007**

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**Table 2 Numbers and transplant rates of allogeneic HCTs for AML and MDS in Europe in 2007**

**Demographics**

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Discussion

Data from this report describe the current state of art of HSCT in Europe in 2007. They document the ongoing role of autologous and allogeneic stem cells for a broad range of malignant and nonmalignant disorders. Allogeneic HSCTs continued to increase, whereas the numbers of autologous HSCT remained within a similar range for most disease indications when compared with the previous years.

For the first time since the introduction of the EBMT activity survey, additional information was collected for novel cellular therapies. They confirm the importance of donor lymphocyte infusions, which parallel the development of RIC for which therapy donor lymphocyte infusion remains an integral part of the therapeutic concept.

More interesting and novel are the substantial number of mesenchymal stem cell grafts and the use of hematopoietic stem cell transplants for nonhematopoietic indications, for example, cardiovascular and neurological disorders, or for tissue repair. No specific information was collected for the donor lymphocyte infusions and the mesenchymal stem cell grafts. It is also clear from some feedback (www.euroct.org) that the numbers of HSCT for non-hematopoietic use as well as of mesenchymal stem cell grafts are probably larger than those reported within this survey. Such grafts are frequently performed outside the traditional hematopoietic SCT units. It will be a challenge for the scientific and political community to collaborate and to get comprehensive information on the numbers, indications and outcome of these grafts. Specifically, mesenchymal stem cells, more correctly called multipotent mesenchymal stromal cells, are being tested in various clinical settings to exploit their proposed antiproliferation and immunomodulatory properties. They are obtained by the ex vivo expansion of stromal cells from BM, cord blood, fat tissue or placenta, and the host does not require conditioning or immunosuppression. Mesenchymal stromal cells actively home to distressed tissue and act through paracrine-secreted molecules rather than by transdifferentiation. The main indications being tested are acute GVHD, myocardial infarct, critical ischemic and autoimmune disease.

Of specific interest is the rapidly increasing role of allogeneic HSCT for AML and MDS. Several prospective controlled trials have confirmed better outcome with allogeneic transplants, and there is often no therapy available for disease eradication other than an allogeneic HSCT. Currently available data are clear concerning an advantage for younger patients with intermediate or high-risk leukemia. The heterogeneity in use of HSCT for AML and MDS, as expressed by the $r^2$ analysis, and the heterogeneity in use in the first CR or at later stages of the disease clearly indicate a need for a continuous evaluation of the technology. This applies even more so for autologous HSCT for AML where the explanatory content is less than 10% (by $r^2$). Data from the survey does not indicate whether a transplant was the best therapy for the individual patient indication or not. Again, it will be the task of the working parties and the AML study groups to provide evidence on the role of HSCT in this clinical situation. Such trials have been initiated (www.ebmt.org).

It is specifically interesting to note that, for the first time since the activity survey began, the numbers of unrelated HSCT equal the number of HLA-identical sibling transplants. There are several reasons behind this development. The massive increase of unrelated donor registries has increased the likelihood of finding a well-matched unrelated donor (www.wmda.org). In addition, there is increasing evidence that the well-matched donor in certain situations might be preferable to a sibling donor, for example, in the situation of an older male patient with the choice between an older female sibling donor and a young well-matched unrelated male donor.

The activity survey does not provide any data on outcome. It also does not provide any information either on the age or sex of the patients or on their pre- or post-transplant therapy. Even more important is that there is no information on nontransplanted patients. Even though desired, this is not the purpose of this data collection. Its key focus is the rapid dissemination of the status quo in the field of HSCT. As such, this activity survey provides a formal basis for patient counseling and health care planning in the field of SCT.
Acknowledgements

The cooperation of all participating teams and their staff (listed in the Appendix), the EBMT Co-ordination office, Barcelona (F McDonald, E McGrath, SM Jones, EF Mac Hale), Paris (V Censes, C Kenfez, NC Gorin), London (C Ruiz de Eivera, S Hewerdine, S de Souza), the Austrian Registry (ASCTR) (H Greimix, B Lindner), the Czech BMT Registry (K Benevska, M Trakova), the French Registry (SFGRM) (D Blainc, E Marry, F Meunil), the German Registry (DRBT) (H Ottinger, K Fuchs, C Muller, S Allguesier, A Siebling, W Neulingger), the Italian Registry (GITMO) (A Bou, R Oneto, B Bruno), the Dutch Registry (HOVON) (A Schattenberg, A. v. Biezen), the Spanish BMT Registry (GETH) (F Carreras, I Espigado, J Lopez, A Cedillo), the Swiss Registry (STABMT) (U Schanz, H Baldomero, E Buhrfeind), the Turkish BMT Registry (G Gurman, M Arat, F Arpaci, M Ertem) and the British Registry (BSBMT) (D Marks, J Cornish, K Kirkland, R Paul) is greatly appreciated. We thank Alan Tyndall for his helpful comments on novel therapies, and also thank S Stoeckl for excellent secretarial assistance and D John for database support. This study was supported in part by the European Leukemia Net (ESN-2002-2.2.0-3, by a Grant from the Swiss National Research Foundation, 3200B-118176, the Swiss Cancer League, the Regional Cancer League and the Horton Foundation. EBMT is supported by grants from the corporate members: Agen Europe, F Hoffmann-La Roche Ltd, Gilead Sciences UK, Milttenyl Biotec GmbH, Schering-Plough International Inc., Celecgen International SARL, Genzyme, Viralharma Europe, Chugai sao—ventis, Fresenius Biotech GmbH, Gambro BCT, Bayer Schering Pharma AG, Therakos, Bristol Myers Squibb, Cephalon, Pierre Fabre Médicament, Alexion Europe, Pfizer, Biosoat SA, Merck Sharp and Dohme.

Conflict of interest

The authors declare no conflict of interest.

References

7 Giordano A, Galdieri U, Marino JR. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. J Cell Physiol 2007; 211: 27–35.
APPENDIX 2007

List of transplant centres in 2007

(The first HSCT (total all HSCT), N allogeneic / N autologous first HSCT)

Albania: no report
Andorra: no report
Austria: no report

Algeria (1 team) (147 (149) 103/44)
Alger, Centre Pierre et Marie Curie, CIC 703, R. Hamaladi (147 (149) 103/44)

Austria (12 teams) (319 (360) 145/174)
Graz, Karl Franz University Hospital (hem), CIC 308, W. Linkesch (45 (47) 20/23)
Graz, Universitäts-Kinderklinik (hem, onco), CIC 593, Ch. Urban (13 (16) 9/4)
Innsbruck, Universitätsklinik (hem, onco), CIC 271, G Gattl, D Nachbar (62 (66) 34/28)
Klagenfurt, General Hospital Klagenfurt, D Geisler, M Heitinger (12 (15) 0/12)
Linz, AO Krankenhaus (onco), I Medizin, MA Frisch (4 (4) 0/4)
Linz, AOK der Elisabethinen, Internal Medicine, CIC 594, D Lutz, O Krieger (44 (49) 19/25)
Salzburg, LKA Salzburg (onco), CIC 356, R. Greil (13 (18) 0/13)
Vienna, AKH, Universitätsklinik für Immer Erkrankt I (onco), CIC 227, JIF Greinix, P Kalka (66 (69) 43/72)
Vienna, St Anna Kinderklinik (hem, onco), CIC 528, H. Gadner, C. Peters (31 (38) 20/11)
Vienna, Hannusch-Krankenhaus (hem, onco), CIC 743, E Koller (11 (15) 0/11)
Vienna, Donauklinik, CIC 767, W Hinterberger (3 (3) 0/3)
Vienna, Wilhelminenspital (hem, onco), CIC 828, H Ludwig (15 (20) 0/15)

Azerbaijan: (1 team)
Baku, Azerbaijan Central Clinic Hospital, CIC 186, S Dincer (no report)

Belarus, Republic of (2 teams) (72 (77) 27/45)
Minsk, Belorusian Center (hem, onco, ped), CIC 591, O Alexiukova (35 (35) 20/15)
Minsk, Hospital No 9, N Milanovich (37 (42) 7/30)

Belgium (20 teams) (630 (715) 264/366)
Antwerpen, Stuivenberg ZH, CIC 339, P Zachie (34 (41) 17/17)

Antwerpen/Edgem, University Antwerpen (hem), CIC 596, W Schroyens (36 (41) 18/20)
Antwerpen, AZ Medischhein (hem), CIC 783, R de Bock (10 (10) 0/10)
Brugge, AZ St Jan (hem), CIC 506, D Sellings, A Van Hoof, J Van Droogenbroeck, K Van Eygen (9 (9) 2/9)
Brussels, Institut Jules Bordet and the Children's University Hospital, CIC 215, D Broe, E Sariban, C Dowak, A Forster (50 (61) 25/25)
Brussels, Clinique universitaire St Luc (hem, ada), CIC 234, A Ferrant (42 (48) 18/24)
Brussels, Clinique Universitaire St Luc (ped), CIC 234, C Versylen (11 (11) 6/5)
Brussels, Hôpital Erasme (hem), CIC 596, W Ferramans, A Kentos, M Lambermont, A Dewaele (15 (19) 0/15)
Brussels, Ac Z VUC University Hospital (hem, onco), CIC 650, B Van Camp, A Schats (25 (28) 6/20)
Charleroi, Hôpital Notre-Dame (hem, onco), CIC 349, M André (17 (19) 4/13)
Charleroi, Hôpital Vésale de Charleroi (hem), CIC 804, A Triffet (5 (5) 0/5)
Cant, University Hospital (hem, ada, ped), CIC 744, LA Noens (35 (37) 16/19)
Hain St Paul, Hôpital de Jolimont (hem), CIC 234, A Delannoy, C Ravot, N Streetmans (17 (20) 2/15)
Hasselt, Vrije Jees Ziekenhuis (hem), CIC 632, G Vlaerstraeten, G Bries, V Madoc (30 (33) 0/30)
Leuven, University Hospital Gasthuisberg (hem, ada, ped), CIC 209, J Maertens, D Dierickx, M Renaud (117 (123) 82/39)
Léé, CHU de la Citadelle (hem, onco), CIC 353, S Van Steenweghein, C Andrez, F Sorbo (6 (10) 0/6)
Léé, University Hospital Sart-Tilman (hem), CIC 726, Y Bégain, B De Prijck (71 (87) 34/57)
Koeskide, Heilig Hartziekenhuis (hem, onco), CIC 646, F Van Aelst, J Tytgat, J Domoil (11 (14) 3/8)
Wrijik, Sint Anastasios GVA (hem), CIC 715, J Lemmens (11 (11) 0/11)
Yvoir, Clinique universitaire Mont-Godinne (hem), CIC 254, C Doyen (25 (30) 6/19)

Bosnia-Herzegovina: (2 teams) (7 (7) 0/7)
Sarajevo, Clinical centre University Sarajevo (hem), CIC 198, A Sofo-Hasicovic (5 (5) 0/5)

Tuzla, University Clinical Centre of Tuzla (hem), CIC 647, M Malesevic (2 (2) 0/2)

Brazil (1 team) (151 (165) 98/53) (not included in analysis)
Jau, Anuaral Carvalho Hospital, CIC 180, C Vergilio (151 (165) 98/53)

Bulgaria (2 teams) (24 (26) 9/15)
Sofia, Pediatric Hospital for Oncohematology and Bone Marrow Transplantation (pedi, hem, onco), CIC 346, D Bobev, B Avarovna, R Yordanova (24 (26) 9/15)
Sofia, National Centre of Hematology and Transfusiology BMT, CIC 859, G Mihaylov (0 (0) 0/0)

Croatia (2 teams) (121 (127) 37/99)
Zagreb, Clinical Hospital "Merkur", CIC 159, B Jakic, H Minigo (27 (30) 6/21)
Zagreb, Clinical Hospital Center, CIC 302, B Labar, D Nemec, M Mrlc (94 (97) 26/68)

Cyprus (1 team) (4 (4) 0/4)
Nicoria Matarkou Hospital Il (hem), CIC 575, A Papastyphon (4 (4) 0/4)

Czech Republic (9 teams) (461 (530) 197/264)
Brno, Masaryk University Hospital (ads, pedica, hem, onco), CIC 597, J Vorlick, J Mayer, Z Kostick (98 (133) 29/60)
Hradec Kralov, Charles University Hospital (hem, onco), CIC 729, L Jelavay, S Filip, M Blaha (88 (4) 22/16)
Olomouc, University Hospital (hem, onco), CIC 574, K Indriek (56 (66) 22/37)
Pilsen, Faculty Hospital (hem, onco), CIC 718, V Kozza (91 (98) 39/52)
Prague, Clinical Haematology, Charles University, CIC 318, T Kozak (29 (29) 0/29)

Bone Marrow Transplantation
Prague, Thomayer Memorial Hospital, CIC 375, J Abrahamové, J Népotová (5 (3) 0/3)
Prague, University Hospital Motol (peds, hem, onco), CIC 452, P Sedlacek (28 (28) 22/4)
Prague, Institute of Hematology and Blood Transfusion, A Vltek, P Kobylika CIC 656 (64 (67) 63/1)
Prague, Charles University, CIC 745, M Tmezy (53 (65) 0/53)

Denmark (4 teams) 024 (291) 83/171
Aalborg, Aalborg Hospital (hem/chim immunology), CIC 848, J Bæcher, 1 Christiansen (26 (50) 0/26)
Aarhus, Amtsbygden (hem) and Skejby Hospital, CIC 634 + 510, E Segel, B Toelner (57 (57) 0/52)
Copenhagen, Rigshospitalet (hem), CIC 206, H Sneegol (157 (184) 83/74)
Copenhagen, Herlev Hospital (hem) University, CIC 568, N Clausen (19 (20) 0/19)

Estonia (2 teams) 40 (42) 9/31
Tallinn, North Estonian Regional Hospital, K Vahit, T Jogi (19 (20) 0/19)
Tartu, University Hospital (hem, onco), CIC 746, H Everaas, A Kaare (21 (22) 9/12)

Finland (7 teams) 279 (303) 107/172
Helsinki, Children’s Hospital, CIC 219, U Pihkala, S Vetterenroth (28 (33) 20/6)
Helsinki, University Central Hospital, Department of Medicine, CIC 315, T Rautu (99 (99) 62/28)
Helsinki, University Hospital (onco), CIC 833, J Joensuu, R Jaana (12 (12) 0/12)
Kuopio, Department of Medicine, University Hospital, CIC 396, E Juntunen, T Nousiainen (33 (40) 0/33)
Oulu, University Central Hospital (hem, onco), CIC 690, P Koistinen, T Turpeinen-Huhtaniemi (27 (30) 0/27)
Tampere, University Hospital (ads, peds), CIC 635, E Koivunen, T Lehtinen, R Silvennoinen, M Arok (30 (40) 0/34)
Turku, University Central Hospital, CIC 225, K Remes (53 (58) 25/28)

France (72 teams) 3705 (4172) 1251/2454
Aix-en-Provence, CHU Aimé, G Damaj (42 (45) 0/42)
Angers, Centre Hospitalier, CIC 650, N H rasho, S Francis (65 (73) 29/36)
Argencon, Hôpital Visteldropouy (hem), CIC 199, L Sutton (16 (14) 0/14)
Besançon, Hôpital Jean Minjoz et Hôpital St Jacques (ads, peds), CIC 233, P Herve, E Deconchic, P Rohrhich (67 (103) 47/50)
Bordeaux, Hôpital des Enfants (hem, onco), CIC 550, P Note-Carrere (16 (12) 2/8)
Brest, Hôpital Morvan (hem), D Gillet (66 (77) 16/50)
Caen, Centre Hospitalier Regional, CIC 251, O Renaux (45 (53) 17/28)
Columbia, Hôpital Cote de Nacre (hem, onco), P Boudret (2 (3) 0/2)
Caen, Centre Regional Francois Baclesse, C Fructaret (29 (30) 0/20)
Clermont Ferrand, Centre Jean Perrin et CHU Hotel Dieu (ads, peds), CIC 273, J-O Buix, P Deneuve, P Travade (98 (119) 41/57)
Colmar, Hôpital civil, B Astdy (7 (8) 0/7)
Corbeil Essonne, Hôpital Gillette de Corbeil, A Devdas (17 (18) 0/17)
Creliez, Hôpital St Quentin (hem), CIC 252, C Cordier, M Kuentz (30 (35) 28/22)
Dijon, Hôpital St-Esprit, D Callot (68 (76) 0/68)
Dunkerque, Centre Hospitalier (hem), M Wetterwald (no report)
Grenoble, Centre Hospitalier A Michallon (ads, peds), CIC 270, JY Cahn, F Garban, P Drillete, D Planteau (101 (109) 47/54)
Lille, Hôpital Claude Huriez, CIC 277, P Bauters, JP Jouet (91 (101) 55/56)
Lille, Hôpital Jeanne de Flandre (peds), CIC 963, B Brunon (1 (1) 0/1)
Lille, Centre Oscar Lambret (onco, peds), A Dufaille (12 (21) 0/12)
Lille, Centre Hospitalier Saint Vincent, N Camber (21 (24) 0/21)
Limoges, Centre Hospitalier Dupuytren (ads, hem), CIC 977, D Bordeaux, P Turlure (43 (48) 0/43)
Lyon, Centre Léon Bérard, CIC 241, P Biron, T Philip (64 (75) 0/64)
Lyon, Hôpital Edouard Herriot, CIC 671, M Michelot, E Wadlet, A Thiebaut, F Nicolle, J Troncy, X Thomas (67 (75) 54/13)
Lyon Sud (Pierre Bénite), Centre Hospitalier, B Collection (76 (86) 0/76)

Bone Marrow Transplantation
Siena, Ospedale San Giovanni (hen), CIC 321, F Lauria (25 (32) 8/17)
Taranto, Ospedale Nord (hem), CIC 332, P Masse, G Palazzo, B Amoruso (29 (33) 10/19)
Torino, Azienda Ospedaliera S Giovanni, CIC 231, M Felda, F Locatelli (61 (72) 24/37)
Torino, Ospedale Regina Margherita (peds), CIC 305, F Fagioli, E Vassallo (44 (54) 30/14)
Torino, Ospedale Mauriziano Umberto I, IRCRC, CIC 377, M Aglietta, A. Capaldi, T Carnevale (29 (35) 6/23)
Torino, Ospedale S Giovanni (hem), CIC 696, M Boccardo, M Massia, C Tarella, B Benedetto, D Caracciolo, A Pieri (55 (92) 17/38)
Trasice (Lecco), Hospital C Panico, CIC 652, V Pavone (27 (27) 10/17)
Trieste, Istituto per l’Infanzia, Clinical Pediatrica, CIC 525, M Andolina (21 (21) 12/9)
Udine, Policlinico Universitario (hem), CIC 705, R Fanin (93 (110) 52/41)
Venezia, Ospedale Civile Riuniti di Venezia (hem), CIC 502, T Chiesi, M Vesprini, M Chinello (15 (18) 2/13)
Verbania-Pallanza, UOGA Oncologia Medica, Ospedale di Verbania, CIC 355, A Luraschi (5 (7) 0/5)
Verona, Policlinico GIB Rossi (hem, onco), CIC 623+514, P Bareisetti (50 (63) 21/25)
Vicenza, Ospedale S Bortolo (hem), CIC 797, R Rainondi, F Rodighiero (40 (50) 18/22)
Viterbo, ASL Viterbo Ospedaliero Centrale, CIC 210, M Montauro (0 (10) 0/0)
Latvia: (1 team) (20 (22) 2/18)
Riga, Clinic Lineers, CIC 385, S Lejnieces (20 (22) 2/18)
Lebanon: (1 team) (20 (25) 5/15)
Beirut, American University of Beirut, CIC 369, A Bazarbachi (20 (23) 5/15)
Lichtenstein: no report
Lithuania: (2 teams) (93 (104) 49/44)
Vilnius, University Hospital Santarukus Klinikos (hem), CIC 644, A Slobinaitis, I Trosickas (83 (92) 44/39)
Vilnius, University Children’s Hospital (hem, onco), CIC 508, J Rascon (10 (11) 5/3)
Luxembourg: no report
Macedonia: (1 team) (16 (16) 6/10)
Skopje, Medical Faculty (hem), CIC 381, B Georgievski (16 (16) 6/10)
Malta: no report
Moldova: no report
Montenegro: no report
Montenegro: no report
Netherlands: (14 teams) (878 (917) 372/506)
Amsterdam, Academic Medical Center (ads, peds), CIC 247, MJ Korsten, J Zairos (69 (75) 22/47)
Amsterdam, Free University Hospital (hem), CIC 588, GJ Oosmekoppe (122 (126) 50/72)
Amsterdam, The Netherlands Cancer Institute, CIC 976, S Rodenhuis, J Baars (14 (16) 0/14)
Enschede, the Medical Spectrum Twente, CIC 360, Dr Schasffza (25 (25) 0/25)
Groningen, University Hospital (hem), CIC 546, G van Imhoff (72 (80) 11/61)
The Hague, Haga Hospital (Leyenburg), CIC 547, PW Wijermans (34 (30) 0/34)
Leiden, University Medical Center (ads, peds), CIC 203, R Willemze, M Egeler (95 (100) 79/16)
Maastricht, University Hospital (hem, onco), CIC 565, HC Schouten, J Wagstaff (55 (58) 17/29)
Nieuwegein, St Antonius Hospital, CIC 200, D Biets, V Veth, O de Woerd (27 (27) 0/27)
Nijmegen, University Hospital (ads, peds, onco), CIC 237, A Schattenberg, P Hoogerbrugge (113 (120) 63/50)
Rotterdam, Dr Daniel den Hoed Cancer Center, CIC 246, JJ Cornelissen (130 (153) 57/73)
Rotterdam, Sophia Children’s Hospital, CIC 998, R Pieters (no report)
Utrecht, University Hospital (hem, ads, peds), CIC 239, LF Verdonck, NM Wallstraat (106 (106) 73/53)
Zwolle, Isala Klinieken/Sophia Ziekenhuis, CIC 548, M von Marnwick Kooy (15 (15) 0/15)
Norway: (6 teams) (207 (220) 66/141)
Bergen, Haukeland Universitetets Sjukhus, CIC 197, M Sjo (30 (37) 13/17)
Oslo, Rikshospitalet Radiumhospitalet, CIC 235, D Albrechtsen, L Brinch (75 (79) 30/25)
Oslo, Rikshospitalet Radiologhospitalet (onco), CIC 782, G Lauritzen, S Kvaloy (41 (41) 3/38)
Oslo, Ullevaal Universitetssykehus (hem), F Wiskolff, J-M Tangen (26 (26) 0/26)
Trondheim, University Hospital of Northern Norway (hem), IM Dahl (11 (11) 0/11)
Trondheim, St Olavs Hospital, J Hammerstrom, A Waage (24 (24) 0/24)
Poland: (17 teams) (684 (765) 255/429)
Bydgoszcz, Nicolaus Copernicus University (peds, hem, onco), CIC 764, M Wysoki, J Styczynski (21 (21) 8/13)
Gliwice, Medical University (hem), CIC 799, A Heilmann (29 (32) 4/25)
Katowice, Silesian Medical Academy (hem), CIC 677, J Holowicki (136 (158) 77/29)
Krakow, Jagiellonian University (hem), CIC 553, A Skotnicki (43 (45) 8/55)
Krakow, University Children’s Hospital, CIC 507, J Godzik (17 (17) 9/8)
Lodz, Medical University of Lodz (hem), CIC 117, T Robak (24 (26) 0/26)
Lublin, Children’s University Hospital (hem, onco), CIC 678, J Kowalczyk (17 (19) 10/7)
Lublin, University Medical School (hem, onco), CIC 695, A Wronowska, M Wach, A Walter-Croseck, W Legie (37 (46) 8/34)
Poznan, Institute of Pediatrics, CIC 641, J Wachowiak (24 (29) 18/6)
Poznan, K Marcinkowski University (hem), CIC 750, M Komarzicki (66 (68) 17/49)
Warsaw, Institute of Haematology and Blood Transfusion, CIC 693, B Marianska, L Konopka, B Nasilowska, K Halabuda, M Szczepanski (28 (29) 12/16)
Warsaw, Maria Sklodowska-Curie, Centre of Oncology, CIC 800, J Wielgosz (47 (49) 9/47)
Warsaw, Central Hospital Military Medical Academy (hem, onco), CIC 816, P Razpecki, K Sulek, C Szyczynski (33 (39) 4/29)
Warsaw, Central Clinical Hospital (hem, onco), CIC 934, W Wiktorski-Fedczak, A Deputa, M Krokiewicz (61 (55) 20/21)
Wrocław, Lower Silesian Centre for Cellular Transplantation with National Bone Marrow Donor Registry, CIC 538, A Lange (47 (53) 18/29)
Wrocław, Medical Academy (hem), CIC 699, K Kulickowski (19 (20) 6/15)
Wrocław, University of Medicine (peds, hem, onco), CIC 817, A Chybicka (55 (59) 41/14)
Portugal: (6 teams) (308 (347) 92/216)
Coimbra, University Hospital, CIC 164, N Costa (23 (27) 0/23)
Lisbon, Instituto Português de Oncologia, CIC 300, M Abecasis (64 (70) 39/34)
Lisbon, Hospital de Santa Maria, CIC 636, J Alves do Carmo, F de Laurida (49 (56) 17/32)
Lisbon, Hospital de St Antonio dos Capuchos, CIC 826, A Botelho da Sousa (52 (59) 0/52)
Porto, Instituto Português de Oncologia, CIC 291, P Pimentel, F Camplido (92 (98) 45/47)
Porto, Hospital S Joao (hem, onco), CIC 529 (merged with CIC 572), JE Guimaraes, F Principe (28 (37) 0/28)
Romania: (3 teams) (81 (81) 9/72)
Bucharest, Fundeni University Hospital (hem), CIC 296, AD Moieanu, D Colita, C Arion (36 36 3/33)
Targu-Mures, Sectia Clinica de Hematologie, CIC 178, I Benedek (24 24 3/21)
Timisoara, Emergency Childrens Hospital 'Louis Turcanu', Ill Ped Clinic (hem, onco), CIC 174, M Serban, C-Janca (21 21 3/18)

Rueda (17 teams) (405 (405) 129/276)
Ekaterinburg, Regional Hospital no. 1, TS Konstantinova, VA Shanay (26 26 7/19)
Kirov, Research Hematological Institute, TP Zagoskina (no report)
Moscow, Russian Children's Hospital (hem), CIC 694, A Maschen, E Skorobogati, E Pach November 65 39/15
Moscow, Cancer Research Center, KN Melkova (33 40 2/31)
Moscow, Institute of Biophysics, AB Baranov (11 14 0/11)
Moscow, Cancer Research Center (peis, hem, onco), G Mentschik (28 28 10/16)

Moscow, Research Hematology Center of RAS, VG Savichenko (34 34 15/19)

Moscow, Main Military Clinical Hospital (hem), SV Shamsens (18 18 4/14)

Montreal, Clinic of Hematology and Cellular Therapy Transplantation Unit, CIC 540, A Novik (65 65 0/65)
Moscow, City Clinical Hospital no. 38, NA Obolova (no report)
Novosibirsk, Institute of Clinical Immunology, CIC 376, I Lisikov (33 33 1/32)

Samara, Regional Hospital, VA Rossiev (5 5 0/5)
St Petersburg, Clinical Center for Advanced Medical Tech. E Podosteva, V Sokoltenko, O Rysayanayka (no report)
St Petersburg, Research Institute of Hematology, KM Abdakadrov (19 19 2/17)
St Petersburg, State Pavlov Medical University (hem), CIC 725, BV Afanassiev, I Zabarokovskaya (79 84 49/50)
St Petersburg, Leningrad Regional Clinical Hospital, IS Yuzgun (no report)

Yaroslavl, Regional Clinical Hospital (hem), VA Lapin (2 2 0/2)

San Marino: no report

Saudi Arabia (3 teams) (214 (234) 157/57)
Riyadh, King Faisal Specialist Hospital and Research centre (onco, ads, hem), CIC 397:1, M AI Jurf (101 101 54/87)
Riyadh, King Faisal Specialist Hospital and Research centre (peis, hem, onco), CIC 397:2, M Ayas (105 112 99/6)
Riyadh, Armed Forces Hospital, CIC 815, A Alshahel (8 8 4/4)

Serbia (4 teams) (90 (98) 21/69)
Belgrade, Mother and Child Health Institute, CIC 558, D Vujic (16 17 8/5)
Belgrade, Clinical Centre of Serbia (hem), CIC 373, J Bila, D Antic (16 16 0/16)
Belgrade, Military Medical Academy (hem), CIC 582, D Stantastic (46 48 12/54)
Novi Sad, Institute of Internal Diseases, Clinical Centre of Novi Sad (hem), CIC 655, S Popovic (12 14 1/11)

Slovakia (5 teams) (144 (151) 29/115)
Bratislava, Roosevelt Hospital (hem), CIC 333, M Markuljak, E Kralikova (14 14 0/14)
Bratislava, National Cancer Institute, CIC 560, J Lakota (71 71 8/83)
Bratislava, University Hospital (hem), CIC 610, M Mitrak (24 27 9/15)
Bratislava, University Hospital, 2nd Children's Clinic, CIC 683, S Sulkarska, J Horakova, I Bodoova (21 21 12/29)
Kosice, University Hospital LF UP JS (hem), CIC 984, E Tostova (14 14 0/14)

Slovenia (1 team) (69 (97) 25/44)
Ljubljana, University Medical Centre (hem), CIC 640, J Pretnar (69 97 25/44)

South Africa (9 teams) (63 (64) 21/42)
Bloomfontein, Faculty of Health Sciences Free State University (hem), V Louw (0 0 0/0)

South Africa (9 teams) (63 (64) 21/42)
Bloomfontein, Faculty of Health Sciences Free State University (hem), V Louw (0 0 0/0)

Bose Marrow Transplantation

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Spain (66 teams) (1803 (1913) 625/177)
Alacante, Hospital General, C Rivas-Gonzales (15 15 6/15)
Barcelona, Hospital Clinic (hem, onco), CIC 214, E Carreras (55 55 40/55)
Barcelona, Santa Creu I Sant Pau (adults), CIC 260, J Sierra, S Bruet (28 28 17/27)
Barcelona, Santa Creu I Sant Pau (peis), CIC 260, I Badell Serra, N Pau, M Torrent (15 15 8/7)
Barcelona, Hospital Vall d'Hebron, Materno Infantil, CIC 527:1, J Saez de Toledo Lodina (46 50 35/11)
Barcelona, Hospital General Vall d'Hebron, CIC 527:2, A. Julia-Font, E. Sanchez (17 17 8/9)
Barcelona, Hospital Mutua de Terrassa (hem, onco), T Marti (9 9 9/9)
Barcelona, Hospital Universitario Germans Trias i Pujol, CIC 613, J Ribera (57 62 25/32)
Barcelona, Hospital Sant Joan de Deu, CIC 668, J Estella Agudo (13 13 0/10)
Barcelona, Hospital Darni i Reynolds (hem), Institut Catala d'Oncologia, CIC 759, R Duarte Palominos, C Ferrer, J Belanga, A Fernandez (44 44 19/25)
Caceres, Hospital San Pedro de Alcantara, E Pardal (9 9 11/9)
Cadiz, Hospital del SAS de Jerez (hem), CIC 612, A Leon (22 22 5/18)
Cadiz, Hospital Universitario 'Puerta del Mar' (hem), CIC 679, J Gil (8 8 0/8)
Canary Isles, Las Palmas, Hospital Insular (hem), CIC 355, J Gonzalez-San Miguel (12 12 0/12)
Canary Isles, Las Palmas, Hospital Materno-Infantil (hem, onco), J Lados Rojas, A Molinos (2 2 0/2)
Canary Isles, Las Palmas, Hospital Universitario de Gran Canaria 'Dr Negrin', CIC 537, T Molero, R Mataix, C Campo, S Jimenez (22 22 17/5)
Canary Isles, Tenenife, Hospital Universitario de Canarias, H Hernandez Nieto, MT Hernandez Garcia (27 27 0/27)
Canary Isles, Tamariife, Hospital NS De la Candelaria, J Garcia-Talavera, J Breno, P Rio Roor (20 20 0/20)
Castellon de la Plana, Hospital General de Castellon (hem), R Garcia-Boyer (6 6 0/6)
Cordoba, Hospital Reina Sofia (hem), CIC 238, A Torres Gomez (50 54 25/25)
Cruces-Bankaldo, Hospital de Cruces (hem), CIC 393, I Zarrur-Verde, F Floristel (21 23 0/21)
Galakao, Hospital de GalaKao (hem), CIC 975, J Ogunsanjo, K Atuza (11 11 0/11)
Girona, Hospital Virgen de la Nieves (hem), CIC 599, M Jurado Checon (31 31 7/24)
Jaen, Hospital Cuidad de Jaen (hem), A Alcalan (14 14 0/14)
La Coruña, Complejo Hospitalario Juan Canalejo, CIC 361, FJ Batlle, C Ramirez, P Torres, R Gonzalez-Rodriguez, R Varela (35 40 4/51)
Lerida, Hospital Arman de Villanova, J Macia (2 2 0/2)
Lugo, Hospital Xeral-Caque, M Gonzalez-Lopez (7 7 0/7)
Madrid, Hospital de la Princesa (hem), CIC 236, A Figuera, A Alegre (41 42 30/11)
Madrid, Hospital Doce de Octubre, CIC 382, J Lahoz (hem), H Cortes Fonse (onco), J Lopez Perez (peis) (59 64 10/49)
Madrid, Hospital Ramon y Cajal (ads), CIC 615, J Orioizola, J Perez de Oyay, J Lopez, J Garcia Larana (53 46 9/34)
Madrid, Hospital Ramon y Cajal (peis), CIC 615, A Munoz Villa (5 5 3/2)
Istanbul, Yeditepe University Hospital (hem, onco), CIC 519, Y Koc (41 (44) 11/30)
Izmir, Ege University Medical Faculty (pedb), CIC 621, S Kansoy (26 (38) 24/2)
Izmir, Ege University Medical Faculty (ads, hem), CIC 628, S Cagirgan (78 (80) 23/55)
Izmir, Dokuz Eylul University (onco), CIC 688, H Ozcan (20 (21) 4/16)
Izmir, Tulu Atkas Oncology Hospital, CIC 369, F Boyaktas, M Tibo, G Saydam (10 (10) 0/10)
Kayseri, Erciyes University Hospital (hem, onco), CIC 677, A Unal, M Celn (74 (74) 47/27)
Tartu, Karadaglu Technical University (hem), CIC 170, E Ovati (13 (13) 6/77)

Ukraine: (2 teams) (46 (51) 6/40)
Kiev, Kiev City BMT Center, CIC 176, E Karamansch, V Khomenko, I Kornzhova, S Borodkin (32 (36) 0/32)
Kiev, Kiev Regional Oncology Hospital (pedb, hem, onco), CIC 177, S Donetska, O Rybak (14 (15) 6/8)

United Kingdom (51 teams) (2487 (2644) 1061/1456)
Aberdeen, The Royal Infirmary (hem), CIC 344, DJ Culligan (17 (20) 5/12)
Bangor, Gwynedd Hospital (hem, onco), CIC 736, D Edwards (16 (16) 0/16)
Bath, Royal United Hospital (hem), CIC 619, C Knechtli (10 (10) 0/10)
Belfast, Belfast City Hospital (hem), CIC 268, F Jones, TC Morris, P Abrams (43 (43) 9/34)
Birmingham, Heartlands Hospital (hem), CIC 284, DW Milligan (28 (30) 12/16)
Birmingham, Queen Elizabeth Hospital (hem), CIC 387, C Craddock, P Malherbe (143 (147) 61/82)
Birmingham, The Birmingham Children's Hospital (hem), CIC 781, PJ Darbishire (28 (30) 22/6)
Bournemouth, Royal Bournemouth Hospital (hem), Poole Hospital, Dean Cancer Centre and Salisbury District Hospital, CIC 765, S Killick, J Colli (29 (29) 0/0)
Bristol, Royal Hospital for Children (allo, ads, pedb), CIC 386.1, JM Cornish, D Marks (84 (88) 80/4)
Bristol, Avon Haematology Unit (auto), CIC 386.2, R Evely, J Bird (37 (38) 0/37)
Cambridge, Addenbrooke's Hospital (hem), CIC 566, C Crawley, RE Marent, J Craig, H Belecon, T Chapman (88 (78) 21/47)
Cardiff, University Hospital of Wales (hem), CIC 303, KMO Wilson, AK Burnett, JA Whittaker, CH Poynton (50 (53) 22/28)
Cheltenham, Cheltenham General Hospital, CIC 398, E Bundell (14 (14) 0/14)
Coventry, University Hospital and Warwickshire NHS Trust, J Mills (16 (17) 0/16)
Dudley, The Dudley Group of Hospitals NHS Trust (hem), CIC 405, S Fernandes (9 (9) 0/9)
Dundee, Ninewells Hospital (hem), CIC 719, D Macleod (3 (3) 0/3)
Edinburgh, Western General Hospital (hem), CIC 228, PRE Johnson, J Davies, F Scott, PH Riddie, P Shepherd (37 (37) 10/27)
Exeter, Royal Devon and Exeter Hospital (hem), CIC 571, C Rudin (16 (16) 0/16)
Glasgow, Royal Infirmary and the Western Infirmary, CIC 244, IG McQuaker, A Parker T Fitzsimmons (67 (74) 34/33)
Glasgow, Royal Hospital for Sick Children (hem), CIC 707, B Gibson (12 (14) 10/2)

Ipswich, The Ipswich Hospital NHS Trust (hem), CIC 128, N Dodd (7 (9) 0/7)
Leeds, St James's University Hospital, the General Infirmary, Pinderfields Hospital (hem), CIC 254, G Cook, S Kinsey, MC Galvin (107 (109) 37/70)
Leicester, Royal Infirmary (hem), CIC 713, AE Hunter (55 (59) 27/28)
Liverpool, Royal Liverpool University Hospital (hem), CIC 501, RE Clark, A Pettitt (55 (60) 29/55)
Liverpool, Alder Hey, CIC 773, M Casswell (10 (11) 8/2)
London, Hammondsmit Hospital NHS Trust, CIC 205, J Apperley, E Olavarris, E Kanfer, A Rahemtulla, R Syedpo (90 (102) 28/02)
London, Royal Free Hospital (hem), CIC 216, S Mackinnon (45 (65) 58/11)
London, Royal Marsden Hospital (hem), CIC 218, M Potter (152 (164) 72/80)
London, University College Hospital (hem), CIC 224, K Thomson (133 (136) 44/39)
London, Great Ormond Street Hospital, CIC 243, P Veys 65 (75) 37/8)
London, The London Clinic (hem), CIC 263, M Potter, P Gravett (17 (17) 7/10)
London, St George's Hospital (hem), CIC 539, EC Gordon-Smith, S Ball (23 (13) 9/4)
London, Guy's Hospital (hem), CIC 721, M Kazmi (56 (64) 26/56)
London, King's College (hem), CIC 763, A Pagliuca (113 (121) 66/47)
London, St Bartholomew's, the Royal London Hospital, CIC 768, J Garben, J Cavenagh, S Agrawal, T Lister (101 (119) 32/69)
London, St Mary's Hospital, CIC 386, J De La Fressange, JD Cavenagh, S Agrawal, T Lister (16 (16) 16/0)
Manchester, Royal Children's Hospital, CIC 521, R Wynn (28 (29) 21/7)
Manchester, the Royal Infirmary, CIC 601, JA Yin (49 (52) 27/22)
Manchester, Christie Hospital (hem), CIC 780, E Liskosonelou (99 (107) 31/68)
Newcastle upon Tyne, Royal Victoria Infirmary and the Sunderland Royal Hospital, CIC 276, GH Jackson, SJ Proctor, F Taylor, A Cant, R Skinner PJ Carey (100 (107) 51/49)
Nottingham, Norfolk and Norwich Hospital (hem), CIC 391, M Lawes, G Turner (7 (7) 0/7)
Nottingham, City Hospital, CIC 717, N Russell, JL Byrne, AP Haynes, A McMillan (109 (110) 44/65)
Oxford, John Radcliffe Hospital (hem, onco), Headington and Wycombe General, CIC 255, T Littlewood, C Bunch, C Mitchell, CChilten, G Hall, J Wainscoat (72 (74) 28/44)
Plymouth, Derriford Hospital, CIC 823, MD Hamon (52 (52) 9/43)
Salford, Hope Hospital, JB Houghton (5 (5) 0/5)
Sheffield, Sheffield Teaching Hospitals NHS Foundation Trust CIC 778.1, J Snowden, and Sheffield Children's Hospital NHS Foundation Trust CIC 778.2, A Vora (78 (83) 30/48)
Somerset, Taunton and Somerset Hospital, S Bolam, SA Johnson (9 (11) 0/9)
Southampton, CIC Wessex, CIC 704, K Orchard, A Dancecombe, J Kohler (75 (76) 31/44)
Stoke-on-Trent, University Hospital of North Staffordshire (hem), CIC 394, R Chasty (14 (14) 0/14)
Swansea, Singleton Hospital, CIC 554, Skett, S Al Ismael (5 (5) 0/5)
Swindon, Great Western Hospital (hem), CIC 608, NF Blesing, A Gray, S Green, A Koster (12 (12) 0/12

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REVIEW

The US National Marrow Donor Program role in unrelated donor hematopoietic cell transplantation

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The National Marrow Donor Program® (NMDP) is headquartered in Minneapolis, Minnesota, USA. Established in 1986, the NMDP currently operates the world's largest registry of unrelated adult donors and umbilical cord blood (UCB) units. Since its inception, the NMDP has benefited from continuous financial support provided by the US government through a series of contracts and grant awards. This funding has supported a large network of donor centers and the recruitment of millions of potential adult hematopoietic cell (HC) donors. More recently, the federal government has also supported a national registry for UCB units and expansion of the available UCB inventories. Today, the NMDP registry lists more than 6.7 million adult donors and 68,000 UCB units. Seventy-seven percent (5.2 million) of the adult donors and virtually all of the UCB units are fully typed for HLA A, B and DR. An additional 5 million donors are available for search through international collaborations. The NMDP currently facilitates more than 3600 recipients each year totaling more than 29,000 transplants since 1987.

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NMDP history and status

The National Marrow Donor Program® (NMDP) is headquartered in Minneapolis, Minnesota. It was established in 1986 through the combined efforts of families and physicians whose goal was a US national registry of adult, volunteer unrelated hematopoietic cell (HC) donors. Initial funding was provided through a grant to the American Red Cross. The history of NMDP's development has been recently reviewed.

Currently, the NMDP receives contracts from the US federal government to operate the CW Bill Young Cell Transplantation Program. This new program, named after Congressman Young who has been an ardent supporter of unrelated donor HCT and the NMDP, continues congressional support for a national BM donor registry and additionally calls for the development of enhanced capabilities to provide UCB units for transplantation. Funding provided under these contracts allows for the continued recruitment and support of adult donors as well as the collection, processing and storage of UCB units. The contracts further specify requirements for the identification and delivery of HLA grafts, support of patients and the collection of comprehensive donor and recipient outcome data.

In fulfillment of its mission and the government mandates, the NMDP functions as a network of participating organizations that perform the various functions involved in facilitating unrelated donor HC transplantation (HCT). The organizations include donor centers, which currently number 73 and are involved in the recruitment and subsequent management of adult volunteer donors. Donor centers are assisted in their recruitment efforts by 10 recruitment groups, which focus upon targeted recruitment within the US racial and ethnic minority populations. When an adult donor is matched with a recipient and a transplant occurs, the donor will donate either BM or PBSC, depending upon the wishes of the transplant physician. PBSC have been collected since 1999 and currently comprise nearly 70% of adult donor donations. BM collections occur at collection centers, which currently number 97, while PBSC collections occur at apheresis centers, which number 89. Twenty-four CB banks, which collect, store and manage UCB units, are currently participating with the NMDP. Transplant centers, which number 169, are typically based at major universities or large private hospitals. Among the many participating organizations are several headquartered outside the US, including seven donor centers and 43 transplant centers.

To further facilitate the international exchange of HC products, the NMDP has established formal cooperative registry relationships with 26 organizations. The NMDP is an accredited registry of the World Marrow Donor Association (WMDA) and a supporter of Bone Marrow Donors Worldwide (BMDW).

NMDP activity

Each month, the NMDP receives over 1200 search requests for new patients. However, not all of these searches result
BM. These observations led the US Blood and Marrow Transplant Clinical Trials Network (BMT CTN) to initiate a randomized comparison of BM vs PBSC in unrelated donor HCT. The trial has accrued more than 320 donor–recipient pairs en route to its target of 550. Accrual is expected to be completed in 2009. Also shown in Figure 1 is the rapid growth in UCB HCT. Currently, UCB recipients comprise 17% of all NMDP-facilitated transplants. On account of the increasing use of double UCB transplant protocols, UCB units represent 20% of all grafts procured by NMDP.

NMDP donor and cord blood unit recruitment

Not only has the number of transplants continued to increase in the past 20 years, but also the number of unrelated donors available on the registry has risen as well. The NMDP accepts donors in good health between the ages of 18 and 60 years to join the registry, although transplant centers prefer to select younger male donors. The NMDP expected to recruit 350,000 new adult donors in 2007. Increasing emphasis is being placed on recruitment within the US racial and ethnic minority populations; these donors were expected to comprise nearly half of the new recruits in 2007. Each newly recruited donor is typed at the intermediate level for HLA-A, B, and DRB1. Most donors now need only to provide a buccal swab sample for DNA-based testing rather than a blood sample. Buccal swabs from donors are stored for further testing as necessary, and have significantly reduced the effort and cost of donor recruitment.

Most donors join the NMDP Registry as a result of community donor drives, as opposed to donors who are recruited at blood centers. NMDP donor centers and recruitment groups plan and conduct hundreds of drives each month.

Although all newly recruited donors are HLA DRB1-typed at recruitment, there remain about 1.6 million donors who joined the NMDP in the early years that have been typed only for HLA A and B. This group of donors is functionally inactive because analysis shows that 99% of all transplants that NMDP currently facilitates utilize a donor who is selected from the A, B and DRB1-typed pool.

The search process

The process for identifying an unrelated donor or UCB starts with the transplant center. Each transplant center designates a transplant coordinator to be the primary communicator with the NMDP. The coordinator submits a search request through NMDP TRANS Link (described below) or through facsimile (fax) request. The request includes the age and diagnosis of the patient as well as the highest resolution of HLA typing available. This information is used to immediately search the 6 million NMDP donors and for an overnight search of several international registries. NMDP has published recommendations about how to search for adult BM donors, and a similar set of recommendations concerning UCB units is in preparation.
NMCPD provides software to transplant centers (TRANS Link®) that allow real-time searching of the complete NMDP inventory of adult donors and UCB units. TRANS Link produces a donor/UCB list that is sorted with the best of the potential donors/UCB at the top. Within HLA match grades, UCB is prioritized according to total nucleated cell content, whereas adult donors are prioritized by HLA-matching likelihood alone. An NMDP innovation called HapLogic predicts the likelihood of allele-level matching based on calculated HLA haplotype frequencies within major racial and ethnic populations. Currently, HapLogic predicts high-resolution matching at HLA A, B and DRBI, but future release in active development will also consider HLA C and DQ. TRANS Link allows the user to further customize the search results by prioritizing specific HLA loci or donor/UCB characteristics (age, sex, CMV status and so on). Currently, using TRANS Link requires that a software program be installed on the user’s computer that accesses the NMDP databases through the Internet. A future release of NMDP-matching software will allow access through a standard www browser interface (for example, Internet Explorer, Firefox).

Although the NMDP lists more than 1.5 million non-US donors from its participating international donor centers in the upfront TRANS Link search, an additional 5 million non-US donors are accessible only through cooperative registry agreements. Searching for a match among these donors is more cumbersome and difficult. BMDW provides its online search as a convenient way to target registries for further inquiries, so NMDP automatically searches BMDW with every new search submission. Several registries, including NMDP, also automatically exchange donor and UCB search information using the messaging protocol, EMDIS (European Marrow Donor Information System, www.xkrd.de/medis.html). For registries that have not implemented EMDIS, the searching process is largely manual, involving facsimile transmissions and e-mail messages.

The donor search process typically takes 3 months from the time the unit or donor is selected for additional testing to the date the transplant occurs. Some searches have taken as little as 2 weeks, but other searches may involve the testing of several CB units or donors and may take several months.

Research

The NMDP believes that research is essential for improving the outcomes of unrelated donor HCT. Controlling the complications of unrelated donor HCT ( drug-related toxicity, graft failure, acute and chronic GVHD) and preventing disease recurrence are the goals of carefully designed and conducted research projects. In 2004, the NMDP merged its research activities with the International Bone Marrow Transplant Registry (IBMTM) in Milwaukee, Wisconsin. The new research organization was dubbed the CIBMTR (Center for International Blood and Marrow Transplant Research) with a mission to conduct observational and interventional research in transplant outcomes, health services, immunobiology of transplantation and statistical methodologies. CIBMTR and NMDP jointly participate in the operation of the data coordinating center for the US national HCT clinical trials network, BMT CTN. In addition, CIBMTR has created a division for supporting smaller clinical trials to supplement the opportunities available through BMT CTN. Websites that present research information relevant to NMDP are www.cibmtr.org, www.bmtctn.net, www.nmcpdresearch.org and bioinformatics.nmdp.org.

Summary

In summary, the NMDP continues to grow and expand, seeking to facilitate more transplants, to improve the success of transplantation, to increase the number of transplant options available for each patient and to provide the best possible graft at precisely the right moment. These goals will be met through continued recruitment of high-quality HLA-diverse donors and UCB units, through improved processes that facilitate worldwide real-time communications and through a comprehensive program of carefully designed research initiatives.

Conflict of interest

Neither author declared any financial interests.

References

The National Marrow Donor Program with emphasis on the early years

Jeffrey McCullough, Herbert A. Perkins, and John Hansen

Thanks to the pioneer work of Drs Robert A. Good, and E. Donnall Thomas, the latter a Nobel Prize winner for his work in the development of hematopoietic cell transplantation, marrow transplants have been able to cure certain diseases that previously were considered fatal. Appropriate genetic matching between donor and recipient, however, is essential to achieve a safe and effective transplant. Studies in mice in the 1950s and 1960s demonstrated the importance of gene matching for the major histocompatibility complex (MHC) to allow for sustained engraftment of donor hematopoietic stem cells and to minimize the potentially severe consequences of graft-versus-host disease (GVHD). The latter, originally known as wasting disease, was recognized as the major limitation to successful marrow transplantation. Clinical studies beginning in the late 1970s confirmed that the genetic rules of transplantation in mice also applied to humans. Matching for the human MHC, also known as the human leukocyte antigen (HLA) system, was shown to be necessary for avoiding the immunological complication of marrow transplants.

Initially, transplants were restricted to patients for whom an HLA-identical sibling donor was available. Unfortunately, fewer than 30 percent of patients had an HLA-identical sibling; thus, a transplant was not possible for a majority of patients who might otherwise benefit from this lifesaving treatment. Seeking an HLA match from the general population was generally considered futile for several reasons. The HLA system was known to be highly polymorphic, and the chance of finding HLA matches among the population at large was estimated to be very unlikely. More importantly, no system existed for recruiting donors, for HLA typing, or for oversight of the large number of volunteers necessary to meet the needs of the many potential transplant candidates. The development of the National Marrow Donor Program (NMDP), launched in 1986, has played a major role in demonstrating that marrow from an unrelated donor can be successfully used, provided that a close match for HLA antigens exists. Today more than 5 million volunteers have their HLA types listed in the NMDP database ready to donate their hematopoietic stem cells to someone unknown.

Robert Graves, a Colorado rancher and doctor of veterinary medicine, is largely responsible for the existence of the NMDP. In 1979, Dr Graves' daughter Laura received a marrow transplant for leukemia from an HLA-matched unrelated donor. Laura's leukemia had initially responded to conventional chemotherapy, but with subsequent recurrence, a marrow transplant was the only remaining option for saving her life; unfortunately, no one in the family matched. Refusing to give up hope for Laura, Dr Graves and his wife Sherry began exploring the possibility of an unrelated donor transplant.

Dr Graves arranged a visit with Dr John Hansen of the Puget Sound Blood Center and Fred Hutchinson Cancer Research Center (FHCRC) in Seattle, Washington. Dr Hansen was a specialist in HLA genetics and was known for his studies of HLA matching in bone marrow transplantation. He advised Dr Graves that unrelated donor transplants had been attempted in only a small number of cases in the early 1970s, but he also explained that important advances in HLA typing and matching had occurred during the intervening years. Dr Graves was sufficiently well prepared and informed to understand that Laura's HLA type was probably the most common HLA type found in the general population. He asked FHCRC to perform a transplant if an HLA match could be found. Dr Hansen conferred with colleagues at the Cancer Center and then began a search of the laboratory HLA research file. Fortunately, one of the research donors who was a Cancer Center employee was found to be an HLA-matched individual, and she agreed to be a marrow donor...
for Laura. In September 1979, following high-dose myeloablative chemotherapy and total body irradiation, 10-year-old Laura became the first patient with leukemia to undergo a marrow transplant from an unrelated donor, and a sustained donor cell engraftment was achieved with minimal side effects. Unfortunately, Laura’s leukemia relapsed 2 years later. Despite this disappointing outcome, her initial complete recovery from the transplant demonstrated the feasibility of the procedure. Her success prompted other parents from across the nation to contact Dr. and Mrs. Graves with requests to help them find a donor for their children. Encouraged by the need, Dr. Graves appeared before Congress multiple times to request funds for the establishment of a national registry of HLA-typed unrelated marrow donor volunteers.

Regardless of the initial demonstrations that successful marrow transplantation between unrelated HLA-matched individuals was possible, very little had been done to address the basic ethical issues on whether it was appropriate or reasonable to ask unrelated individuals to become marrow donors. In 1981, Dr. Jeffrey McCullough organized a conference to discuss the scientific, ethical, legal, financial, and practical issues of developing a volunteer marrow donor program as well as developing a framework for approaching community volunteers about marrow donation. The conference achieved clarification about the ethical approach to seeking volunteer marrow donations, and an important consensus was reached about the propriety of recruiting volunteers for a marrow registry. It was an essential milestone for those patients, families, and physicians who were desperately advocating unrelated donor marrow transplants.

One of the initial issues at the conference was to obtain a better understanding of marrow donation risks. Two comprehensive reports on the complications of marrow donations compiled data from several thousand individuals who had donated. From this information, it was then possible to develop a structured medical evaluation of potential marrow donors, although the difficulty in predicting the risks of general anesthesia in normal adults who would otherwise meet the medical eligibility criteria for marrow donation remained an issue.

Legal issues were also addressed, which raised a large number of potential concerns and problems. The discussion focused, however, on the view that the legal system should not be perceived as a barrier to this scientific and medical advancement as long as proper analysis and forethought were provided to the donor assessments and the consent process. Also dealt with were financial issues, which included allocating the donor’s medical costs of donation to a charging system for the patient, providing a life insurance policy for the donor, establishing a system to assure confidentiality and donor anonymity, and preventing the donor from making financial demands on the recipient.

The process of resolving these and many other issues led to the development of a set of operating principles, which later became the initial policies for the operation of the NMDP. These principles addressed the need for future studies on the effects of the request and donation process on donors. Several subsequent reports indicated that donors, when properly approached using the careful strategies designed by these donor programs, were quite willing to donate and did not experience adverse psychosocial effects. Thus, these early experiences with local marrow donor programs formed an excellent basis for the development of a large, multicenter national program.

Subsequent to the 1981 conference, the Blood Center of Southeastern Wisconsin recruited its first unrelated marrow donor from plateletpheresis volunteers, and in 1982, the American Red Cross Blood Center in St. Paul, Minnesota, established a similar program to provide unrelated marrow donors. These programs established the possibility of obtaining volunteer marrow donors through donor-based organizations that were not part of a transplant program.

These successes in finding willing donors substantiated Dr. Grave’s requests to develop a national center. After Dr. Graves obtained the help of Senator Paul Laxalt from Nevada, the National Organ Transplant Act of 1984 included language about marrow transplantation using unrelated donors. With this achievement, Senator Laxalt wrote a letter to the Secretary of the Navy recommending that the Navy, which had an existing marrow transplant research program, establish a national registry. On November 30, 1984, during the American Society of Hematology’s annual meeting in Miami, Captain Jim Woody (USN) called a meeting to discuss essential characteristics of a national registry and to consider the composition of a Request for Proposals (RFP). This RFP would be directed to institutions or groups capable of contracting to establish a national registry of marrow donors. Although the development of an RFP was an essential requirement for any future public funding, there remained significant obstacles to developing a functioning national registry.

The 1984 National Organ Transplant Act had directed the Secretary for Health and Human Services to study the feasibility and effectiveness of a national donor registry, and in May 1985, a Technology Assessment Meeting was held under the auspices of the National Institutes of Health. The panel concluded that although continuing efforts to use unrelated donors were certainly warranted, it was premature at that time to commit to a centralized national marrow donor registry. They suggested the establishment of a coordinating center to facilitate communication among regional registries, however, which were encouraged to continue and expand. The panel believed that the coordinating center should respond to requests.
from transplant centers and refer those requests to the regional registries.

On the assumption that the regional registries discussed by the Technology Assessment Panel corresponded to the donor centers visualized for the National Marrow Donor Registry, in early 1986 Senator Luault was able to attach an appropriation of $1.2 million to the Defense budget, which directed the Navy to establish a registry. With knowledge of the funding available and the Navy's plans to release an RFP, Dr Graves went to the boards of the national blood bank organizations hoping to secure their cooperation and support. These organizations represented multiple blood centers that could promote a national network of dedicated, professional facilities for donor recruitment and management as well as a strong, independent base for donor advocacy. Furthermore, the blood banks were well positioned to make two major contributions. First, they had access to donors of platelets (PLTs) by apheresis—individuals who had already demonstrated a willingness to take additional time and undergo discomfort to benefit a patient they did not know. Second, many of those PLT donors were partially typed for HLA antigens, which helped solve the dilemma of the lack of funds available for a HLA typing program. The HLA types available from PLT donor volunteers could be used to begin a search for a compatible donor; additional HLA typing could be done as needed, with the cost charged to the patient who requested the typing. It was agreed that the American Red Cross (ARC), American Association of Blood Banks (AABB), and the Council of Community Blood Centers (CCBC, now known as America's Blood Centers) would sponsor the marrow donor registry with the ARC taking administrative responsibility.

The ARC appointed Dr Jeffrey McCullough as its representative and proposed that the registry be established as part of its St. Paul center. The AABB appointed Dr Herbert Perkins, Scientific Director of the Irwin Memorial Blood Bank in San Francisco, California, and the CCBC appointed Dr John Hansen, from the Puget Sound Blood Bank and the Fred Hutchinson Cancer Research Center in Seattle, Washington. In addition to his role in Laura Graves' transplant, Dr Hansen had also accompanied Dr Graves on a number of trips to consult with members of Congress. Dr McCullough agreed to become Principal Investigator (PI) for the project, and together with Drs Hansen and Perkins, he began to assemble a plan and written proposal in response to the Navy RFP. The application was successful, and the Navy awarded the first contract for a national registry to the three sponsoring organizations. The contract became operational on July 1, 1986, and the National Bone Marrow Donor Registry established its National Coordinating Center within space provided by the St. Paul Red Cross Blood Services.19

The three investigators met frequently over the next months, accompanied by Dr Graves in his preferred role as the unofficial power behind the throne, to focus on the tasks necessary to complete the setup of the Registry. Staff was hired, a search algorithm was defined, and a subcontract was established with the biostatistical center at the University of Minnesota to provide data management and computer support. On October 28, 1986, a meeting of marrow transplant physicians and other experts was convened in St. Paul to establish by consensus membership criteria for participating transplant centers, indications for transplantation, and minimal requirements for HLA matching.

From the beginning, research was determined to be a major priority for the NMDP. Proof that unrelated donor transplants were sufficiently successful to warrant their routine use required agreement from the transplant physicians to report details of the courses of their transplanted patients to the NMDP. Subsequent research focused on the unique problems of unrelated donor transplants and how to achieve best results. A major focus has been on the role of HLA matching in this situation, and to meet that goal a repository of cells from the donors and recipients was established, which has permitted retyping of the donor-recipient pairs as histocompatibility testing improved. The repository contract was given to the Irwin Memorial Blood Bank in San Francisco because of its prior experience in long-term frozen storage of cells under sterile conditions.

The Navy contract's requirement to establish a Board of Directors was accomplished by Dr Graves and the three investigators. The Board's first meeting occurred in Minneapolis on January 15, 1987, where Dr Graves was elected to Chair and Admiral E.R. Zumwalt, Jr, former Chief of Naval Operations, was elected as Vice Chair. Other members of the original board included Walter Mondale, former Vice President of the United States; Armand Hammer, philanthropist; Dr David Rogers, Professor of Medicine at Cornell Medical School and former President of the Robert Wood Johnson Foundation; David Frohnmayer, Attorney General of the State of Oregon; Dr Bo Dupont of Memorial Sloan-Kettering Cancer Center and a major investigator in the field of HLA typing; Dr Arthur Caplan, Hastings Institute and distinguished medical ethicist; Dr E. Domall Thomas; and Dr Robert A. Good.

Almost immediately, concern arose about further financial support; the original appropriation funded the program for only 15 months. On January 25, 1987, Drs McCullough, Perkins, and Hansen were invited to a meeting on the NIH Bethesda campus involving representatives from the Office of Naval Research (ONR) and the National Institutes of Health to discuss funding for the NMDP. Senior ONR officials were reluctant to accept the responsibility for further funding, and National Institutes of Health officials considered the project inappropriate and of low priority for research support. With the efforts of Dr Graves, however, along with Admiral Zum-
walt and Mr Bart Fisher, funding from Congress for the NMDF continued. Mr Fisher, a Washington attorney who later joined the NMDF Board, had lost a son from aplastic anemia. A marrow transplant had been recommended, but no HLA-matched donor could be found.

In September 1987, the NMDF began accepting requests for donor searches. The first donor was provided in December 1987—a dramatic story chronicled in the *Readers Digest*. A fierce winter storm had closed the Milwaukee airport, and only the bravery of a private pilot in a small airplane made it possible to transport the marrow from Milwaukee in time to be given to a distant patient.

Over the next year, the number of transplants increased steadily and by February 1989, it was considered a remarkable achievement when marrow from an unrelated donor was delivered to the 100th transplant recipient. The White House commemorated this event by holding a celebration for the members of the board and staff hosted by John Sununu, the President's Chief of Staff.

Meanwhile, the recruitment of potential donors was vastly accelerated by the activities of families with children who needed a marrow transplant but found no compatible donor in the files. Early efforts by the family and friends of Allison Atlas recruited more than 70,000 donors, focusing on results on donors of similar ethnic background (Ashkenazi Jews), and the family and friends of Joanne Johnson recruited many tens of thousands of African-American donors. Community drives have continued to dominate NMDF's recruitment accomplishments, providing 75 percent of the donors in the Registry. Several large marrow donor registries that had been started independently of the NMDF became donor centers within the organization including the Heart of America (organized by Fred and Sandy Harris of Kansas City) and the New Jersey HLA Registry (a project of Dr Elie Katz). An additional mechanism for recruiting donors with emphasis on minority groups came with the establishment of recruitment groups targeted to specific populations. The first such recruitment groups to be formally accepted into the program were the Judy Davis African-American Marrow Donor Program and the Asian-American Donor Program, both located in Oakland, California. Along with the vigorous activities to enlarge the donor population, considerable effort was devoted to defining the risks of marrow donation and using this knowledge to establish criteria for the medical evaluation and suitability of potential donors.

The increasing growth of the program created a need for a full-time director of the Coordinating Center, and in April 1989, Douglas Shaw was hired as Executive Director. The three original investigators of the Navy registry contract were elected to the NMDF Board, with Dr McCullough serving as President, Dr Hansen as Secretary, and Dr Perkins as Treasurer.

Although Congress continued to allocate increasing amounts of funding to the NMDF through the Navy, the success of NMDF and increasing need for unrelated donor transplants created an inevitable requirement to move Federal oversight of the NMDF from the Navy to another Federal agency more appropriate for an emerging major domestic health care program. Congress subsequently redirected authority for the National Registry through funding for the Organ Transplant Amendments Act of 1986, which reauthorized funding for the NMDF with instructions to the Secretary for Health and Human Services to establish a national registry. In 1988, the Secretary assigned the oversight responsibility to the National Heart, Lung, and Blood Institute (NHLBI). The Navy, however, continued to provide the NMDF with additional funds to supplement selected program activities, especially minority donors and HLA research. The largely responsible for ongoing program support is Congressman Bill Young of Florida. Congressman Young has demonstrated remarkable commitment to the NMDF mission, his leadership has ensured continuing congressional support for the national effort to meet the needs of patients seeking transplantation.

In November 1989, Admiral Zumwalt was elected to succeed Dr Graves as Chair while Dr Graves remained as Vice Chair. Over the next six years, the Admiral presided over a series of dramatic changes that shaped the status of the NMDF. He and Dr Graves continued the critical process of building public and Congressional support. Although Dr Graves remained very active as Vice Chair for several more years, Admiral Zumwalt, who had a son who also had needed a marrow transplant, became the dominant figure. Admiral Zumwalt was a widely known and highly respected public figure. He was involved in the negotiations that led to the separation from the Red Cross and the establishment of the NMDF as an independent entity. Operational capability of the NMDF was further established with the transfer of the donor files and biostatistical unit from the University of Minnesota to a new bioinformatics and statistics unit within the NMDF coordinating center. Zumwalt was also highly influential in resisting NHLBI pressure to limit the role of the NMDF Board to that of an advisory group only. He also led the successful effort to integrate Lifesavers (an independent donor recruitment agency) into the NMDF, and he established The Marrow Foundation as a separate nonprofit organization dedicated to raising funds for NMDF programs.

In February 1990, Dr McCullough resigned to devote more time to his university and blood bank responsibilities. With continual program growth, an increasing number of member organizations, and expansion into an international presence, the NMDF no longer found it necessary or adequate to operate under the auspices of the ARC. Therefore, in 1990, the NMDF became a separate,
nonprofit corporation and assumed full responsibility for administration of the federal contract from the ARC. In December 1990, the coordinating center moved from its quarters within the St. Paul Red Cross Blood Center to a freestanding location in Minneapolis. Shortly thereafter, following extensive development of new software programs, the computer services for the registry were transferred from the University of Minnesota to the NMDP Coordinating Center.

The remarkable growth of the NMDP, the establishment of independence from the ARC, and a change in its federal oversight to the NHLBI led to a need for reorganization. On April 5, 1991, the board appointed an ad hoc committee for reorganization chaired by Dave Frohnmayer’s recommendations, which were subsequently adopted by the board. The primary focus of the changes was to provide a Chief Executive Officer (CEO) with stronger management and policy authority. A national search for a new CEO was undertaken, and Craig Howe, MD, PhD, a transplant physician and Professor of Medicine at the Medical College of Virginia in Richmond, Virginia, accepted the new position of CEO of the NMDP in May 1992.

With the Transplant Amendments Act of 1990, Congress further defined and expanded the functions of the program. Establishment of an office for patient advocacy was mandated, and the NMDP was directed to emphasize recruitment of minorities with the goal of providing equal access for patients of all ethnic groups. Subsequently, money from the OHR was made available for recruitment and HLA typing of minority donors.

In 1994, oversight was transferred from the NHLBI to the Division of Transplantation of the Health Resources and Services Administration (HRSA). This was recognition that the use of unrelated donors for marrow transplantation was no longer an experimental procedure but a vital service that needed to be provided to a larger number of patients, as indications for transplantation using unrelated donors became better defined and the chance of finding an HLA match steadily improved.

The NMDP story is not complete without reference to the extraordinary international collaboration that occurred among marrow donor repositories in different countries. The first large registry of potential marrow donors was the Anthony Nolan Registry located in London, which began providing some donors for US patients before NMDP existed. In addition to Anthony Nolan, the NMDP established donor exchange agreements with Australia, Austria, Belgium, Canada, Cyprus, Czech Republic, France, Germany, Hungary, Ireland, Italy, Japan, Portugal, Singapore, Slovenia, Spain, Switzerland, Taiwan, and the United Kingdom. Some foreign donor and transplant centers have elected to affiliate directly with NMDP for ease of access, as fully participating members of the NMDP network, including donor centers and transplant centers in Argentina, Denmark, Germany, Israel, and the Netherlands. Twenty percent of NMDP-facilitated transplants involve foreign donors or recipients and the bidirectional exchange has been approximately equal (D.Confer, personal communication, April 2005). NMDP provides financial support for a publication organized by Dr. J.J. Van Rood in the Netherlands (Bone Marrow Donors World Wide), which lists on the Internet all of the HLA types available from all of the significant marrow donor registries in the world. This publication provides access to more than 9 million donors including the more than 5 million listed with NMDP. The NMDP searches this file if it does not find a matched donor in its own registry (Fig. 1).

When the registry began in 1987 with fewer than 10,000 donors, we set a goal of 100,000 donors. This was based on economics (the amount of funds available for HLA typing) and political reality. We were unsure that there would be sufficient political will and funding to set more aggressive goals. It seemed more important to move quickly to expand the number of available donors so that more transplants could be done to move the field forward. In addition, we could not in the mid-1980s fully anticipate the extent of the polymorphism that became evident with the implementation of DNA typing methods in the 1990s. The registry now totals more than 5,000,000, and each month approximately 27,000 donors are added to the file and a mean of 210 transplants are carried out. Analysis of results published by NMDP-affiliated investigators demonstrates steady improvement in disease-free survival of recipients. Similarly, NMDP-affiliated investigators have carefully monitored and reported on the experiences of volunteer marrow donors and their recovery following marrow donations. Although GVHD is more common in HLA-matched unrelated transplants compared to HLA-genotypically identical sibling donor transplants, improvements in HLA typing and overall supportive care have significantly benefited unrelated donor transplants. For certain patients, overall survival is approaching that achieved with an HLA-identical sibling donor.
Although the NMDP name includes the word “marrow,” its activities have not been restricted to hematopoietic stem cells obtained from marrow alone. After there had been sufficient experience with peripheral blood progenitor cells (PBPCs) using related donors, NMDP began a cautious program of PBPC collection, initially for repeat transplant only and subsequently as a primary source. The concern was for the unrelated donor who had to receive multiple injections of granulocyte-colony-stimulating factor (G-CSF) and then undergo one or two apheresis procedures. G-CSF has uncomfortable side effects, and its long-term consequences are still unknown. NMDP studies of PBPC are conducted under Investigational New Drug (IND) approval from the Food and Drug Administration, and stem cells are collected only at centers that are approved after demonstrating that they meet NMDP standards. PBPCs are increasingly favored over marrow as a source of stem cells and currently about 60 percent of stem cell transplants through NMDP are from peripheral blood.

NMDP has also begun to include umbilical cord blood as a source of hematopoietic stem cells. NMDP lists cord blood from 15 cord blood banks containing approximately 42,000 cord blood samples. NMDP is thus the largest single source to be accessed for unrelated donor cord blood, also through an IND from the FDA. In 2003, 4 percent of stem cell preparations procured through NMDP were cord bloods.

The availability of both PBPCs and umbilical cord blood for unrelated donor transplantation opens an exciting new phase in hematopoietic reconstitution. As of April 24, 2005, 21,245 transplants have been performed through the efforts of the NMDP (Fig. 2), often in far from ideal circumstances. All patients were given a chance for life, which would otherwise not have occurred, and 25 to 75 percent (depending on the disease, its stage, and the age of the patient) are living disease-free.

The NMDP has also had a major impact on the development of HLA typing technology. Faced with the need for a very large volume of initial HLA typing at the time of donor recruitment, the NMDP established competitive contracts with typing laboratories at greatly reduced prices. Quality control procedures were initiated to monitor and provide HLA typing accuracy. In addition, the NMDP promoted the development of molecular typing technology as a way of overcoming the inaccuracies in serologic HLA typing. Their aggressive management of donor HLA typing was instrumental in advancing adoption of molecular typing, accelerating progress by years. An extremely low discrepancy rate now occurs in typing, as evidenced by ongoing monitoring by the NMDP, which sends blinded samples of known types to its contract laboratories. More recently, the NMDP has made high-resolution HLA typing needed for final donor selection available on a rapid 7- or 14-day turnaround schedule. This option has been essential for facilitating rapid donor workups for patients with urgent clinical conditions.

As already stated, one of the major goals of the NMDP from the beginning was to carry out research to prove the benefit of unrelated donor transplants and to identify ways to improve results. Transplant centers are required to report periodically on the results of their transplants and the clinical conditions of their patients. The repository of cells from the donor and recipient of each transplant has permitted updating their typing with high-resolution DNA techniques, providing the opportunity to relate HLA mismatching to clinical results. Currently, 198 individual studies approved by the NMDP Research and Publications Committee and/or its Histocompatibility Committee are in progress. An accelerated research program is anticipated since the NMDP has become an active participant of an NIH-organized and-funded clinical marrow transplant trials network (http://spitfire.emmes.com/study/bmtn/) and also has joined with the International Bone Marrow Transplant Registry to create a unified and coordinated research program that will increase the capabilities and productivity of both organizations.

In conclusion, the most important and primary accomplishment of the NMDP has been enabling more than 20,000 patients to receive a potentially lifesaving transplant, but this story of NMDP’s early days carries lessons far beyond this limited area. It demonstrates that one man can make a difference, that persistence in pursuing a valuable goal may be required to achieve success, and that the success obtained may far exceed the initial expectations of the investigators. The program has shown that very large numbers of individuals will commit to an uncomfortable and possibly risky procedure for the benefit of someone they do not know and that very large numbers of ordinary people will devote many hours and significant cash donations to help a procedure that has the promise of saving lives. It supports the old truth that success breeds success.
The program has demonstrated the value of considering the ethical implications of procedures, especially those involving unrelated volunteers and of the need to include patients, their families, and ethicists into the decision-making process.

Without the continual financial support from federal agencies from funding mandated by Congress, the program could not have achieved the success it has reached. Persistent lobbying and the establishment of committed friends in Congress have been essential, and transplant physicians, patients, and families have played a necessary role in obtaining this cooperation. Dr. Graves always tried to bring with him a patient whose life had been saved by a marrow transplant whenever he needed support from an agency.

The program has also demonstrated the possibility and benefit of cooperation among organizations. The program could not have been established without joint sponsoring of the American Red Cross, the AABB, and the Council of Community Blood Centers. Although these sponsors no longer play a direct role in NMDP activities, their importance to the start of NMDP cannot be overestimated. The program’s success led to the incorporation of previously independent donor centers, thus instituting a single-central source to which transplant physicians can turn when they need an unrelated donor, allowing them to select the best donor of marrow, PBPCs, or cord blood.

The large size of the program has permitted it to control the costs of HLA typing and to promote and monitor its accuracy.

The program’s growth and accomplishments have vastly exceeded the optimistic hopes of its founders, and contrary to expectations, the rate of donor recruitment and of transplantation show no signs of slowing down.

For further information about the National Marrow Donor Program please refer to the NMDP Web site.10

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CHAPTER 7
SUMMATIVE COMMENT
UNPUBLISHED AND ONGOING WORK
**Perspective and justification**

Each of these active studies is continuing to generate new and relevant data relating to the central research theme. Superficial reflection might suggest that they are extraneous to the overall scope of this thesis whereas counter-argument highlights the need to capitalise on achievement in sustaining growth and utilisation of these often life-saving interventions. Emphasis, and indeed urgency, is found in the relentless contraction of specialised programs that enjoy international designation as centres of excellence. Acceptance of responsibility to safeguard these is reflected in the decision by the Faculty of Health Sciences at Stellenbosch University to bring at least one such facility formally within their academic ambit. Accordingly concepts and preliminary results generated under these circumstances be specifically included with the caveat that they are positioned in this separate chapter so giving focus to such somewhat limited information as may be available from developing countries²³,⁷⁴,⁸⁰,²¹³-²¹⁶.

Three broad considerations exist

**Immunologic reconstitution**

Little doubt exists that, once the immediate danger of haematologic cellular recovery has been successfully navigated, substantial risk persists from impaired defence against environmental pathogens that can reach life-threatening proportions. Additionally there is an increasing appreciation of other adverse events, exemplified by late rejection and graft-versus-host disease, continuing to occupy centre stage in determining both survival and quality of life. To further explore this important facet of post-transplantation pathophysiology two phases are projected. A reference group of individuals are to be characterised and could reasonably be designated as the learning set using appropriate laboratory measurements matched to clinical status. In parallel what might be regarded as a study or training set will serially examine the same parameters as a function of time. In context both innate and adaptive arms of our natural protective system be correlated with patterns of infection on the one hand and the spectrum of recipient tissue responses on the other. In sub-analysis relate these phenomena to different types of transplant across disease categories that will include both children and adults. Viability is to be vested in a collaborative masters project²¹⁷-²²⁰.
**Survivorship - a concept linking cure with care**

As life after stem cell grafting continues to extend it has become evident that late-presenting side-effects have been largely under recognised. This is a realisation of major importance and being actively and systematically evaluated in association with the PhD student. The experimental design rests securely on personal observations over more than a decade that have revealed, often subtle, tissue and organ injuries in pulmonary parenchyma, cardiac myocytes, both osteoclastic and osteoblastic skeletal integrity, gastrointestinal tract and skin. As experience accumulates treatment programs undergo continuous modification seeking to confer immediate benefit to recipients. Intellectually advances are integrated into outcome analysis adding a relevant further dimension to the more traditional end point of only survival\textsuperscript{221-224}.

**Current status and applicability of this study to an emerging South Africa**

Three consecutive but inseparable phases characterise the global project.

Firstly, as the starting point, experimental and developmental haematology was employed to standardise the fundamental technological aspects. There followed systematic translation into clinical programs that were constantly refined and upgraded to comply with world expectations. Procedures ranged from autologous reinfusion to various forms of allogeneic grafting with the initiating centre being at the University of Cape Town in Groote Schuur Hospital. Provision was made for all patients, without exception, throughout South Africa. New innovations were introduced and the entire project under constant supervision being accredited by international registries.

Secondly arose the appreciation that, as evident particularly in Europe and United States of America, a single centre wherever located, was somewhat restrictive in the light of emerging national requirements. Accordingly was the challenge to create a matching facility in the private sector. 15 years later the venture has clearly emerged as not only sustainable but entirely realistic thereby creating a precedent that has subsequently percolated widely throughout other parts of our country.
An unvarying theme has remained accountability reflected in a 30 year unbroken record of accreditation by worldwide registries with designation as a transplant, harvest and donor centre. Within this framework the successful establishment of a South African Bone Marrow Registry so ensuring access to matched unrelated volunteer donors from other participating countries.

Thirdly has been sustained leadership by undertaking the inaugural survey, with presentation and publication of outcome, for these activities in South Africa. This further imperative is similar to the exercise conducted by the European Bone Marrow Transplant Registry. It is anticipated that there will be two consequences. One that some degree of resource allocation should then be possible with properly performing centres enjoying endorsement on academic rather than the more popular incentive driven criteria. The other increasing commitment by interested investigators to undergo registration by international harmonisation mechanisms offered by The Joint Accreditation Committee of International and European Transplant Groups known as JACIE and the Foundation for the Accreditation of Haematopoietic Cell Therapy or FAHCT so demonstrating compliance with accepted standards of practice. A particularly desirable consequence of meeting these two important responsibilities is an opportunity for the Department of Health to impose some sort of long overdue, but hopefully rational, control as soon as the necessary regulations have been promulgated and registration introduced in the broad context of blood transfusion and human tissues act.

The simplification of the SCT procedures in developing countries has resulted in cost-lowering and availability to more patients.

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In order to simplify and cutting down expenses of the bone marrow transplantation procedures we have made some efforts, trying to provide the best transplantation technique for the individual patient with scarce resources. In relation with the source of stem cells, we have found very attractive the use of peripheral blood stem cells (PBSC) instead of bone marrow cells. This procedure can produce substantially more rapid engraftment than observed with bone marrow. The rapid hematopoietic reconstitution in the recipient of PBSC lower expenses stemming from the use of antibiotics, blood bank products and days at the hospital. In Mexico, a country with a large number of cases of severe aplastic anemia we decided to use PBSC to transplant 10 patients with this disease, in order to take advantage of the large number of stem cells that this procedure can provide. We had excellent results in these patients, avoiding the high cost of the conventional transplantation and the use of ATG. In relation to autologous transplantation we have made efforts to perform autografts without the use of freezing devices, keeping the stem cells in liquid form using a conventional blood bank refrigerator for up to 96 hr. This modifications have resulted in diminishing costs and increasing the availability of the procedure to a large number of patients. Finally in Mexico we have had experience with non-myeloablative transplants, and obtained reasonably good results, with a median cost of 18,000 USD per allogeneic transplantation procedure. In some cases this is the only affordable therapeutic option. In developing countries where very few patients can afford the cost of conventional bone marrow transplants, any reasonable effort in the direction of simplification must be welcome.

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Hematopoietic stem cell transplantation for autoimmune diseases in developing countries: current status and future perspectives

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Summary:

In this paper we present preliminary results of hematopoietic stem cell transplantation for autoimmune diseases in Brazil and China. Chinese experience transplanting lupus is significant and the Brazilian experience with several autoimmune diseases is growing. We discuss peculiar conditions in developing countries which could affect the results, and future perspectives for the organization of phase III randomized trials in those countries.

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Keywords: hematopoietic stem cell transplantation; autologous; autoimmune diseases; systemic lupus erythematosus; multiple sclerosis; developing countries

Application of hematopoietic stem cell transplantation (HSCT) for the treatment of systemic autoimmune diseases (AID) in developing countries requires consideration of peculiar aspects of the diseases and of the health system in those countries, including: (1) Probably a worse activity and prognosis of severe AID in the patient population treated with conventional therapy due in part to poor economical and social conditions.¹ (2) High prevalence of some autoimmune diseases such as rheumatic fever and some forms of pemphigus in Brazil and of systemic lupus erythematosus (SLE) in China (70/100,000). (3) Difficult access to new technologies (stem cell selection columns, monoclonal antibodies, etc) and therapies (anti-TNF agents, new immunosuppressive drugs), which impairs the capacity to deliver the best medical treatment to the patients and to participate in international cooperative trials. (4) Universal coverage of health care by the state, but in a highly regulated fashion for high-cost therapies such as HSCT. (5) Large availability of HLA-identical donors (>50%) in the general population in some countries, like Brazil² but not in others, like China. In this work we present preliminary results of HSCT for AID in two big developing countries, Brazil and China, and discuss characteristics and perspectives of the programs, which could apply to other developing countries.

HSCT for AID in Brazil

Bone marrow transplantation centers in Brazil have a large experience transplanting hematologic autoimmune diseases, such as acquired aplastic anemia, including the design of new conditioning regimens for hypertransfused/presensitized patients,³ and anecdotal cases of systemic autoimmune diseases submitted to autologous HSCT have been reported since 1996 with favorable results.⁴

In October 2000, a meeting was organized in Ribeirão Preto, Brazil, to launch a program of HSCT for autoimmune diseases in the country. International experts from USA (Richard Burt, Dhaval Patel, William Burns) and Europe (Rezae Arnold) met with representatives of main BMT/Rheumatology/Neurology Brazilian groups and it was decided to start a pilot, phase I/II national cooperative study of autologous transplantation for refractory systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and multiple sclerosis (MS). Mobilization of HSC is performed with cyclophosphamide (2g/m²) plus G-CSF (10μg/kg/day) and conditioning is CY + ATG for SLE, BEAM + ATG for MS and CY + Fludarabine + ATG for SSc. Horse ATG is given at 15mg/kg/day (three doses before stem cell infusion and three doses after infusion) to replace in vitro T-cell depletion/SC selection.⁵ Conditioning for SLE and MS followed standard protocols used elsewhere,⁶ while for SSc a highly immunosuppressive combination was adapted from mini-allo transplants,⁷ The program started in June 2001 and eight transplants have been performed under the protocol (four for SLE, three for MS and one for an overlapping syndrome of SLE + SSc) in three centers, and other centers are obtaining IRB approval and accruing patients to be engaged in the protocol. Preliminary results show beneficial effects in most patients (three SLE and two MS patients) and an initial significant morbidity/mortality of the transplant procedure because of specific problems of the patient group (kidney failure and fluid overload in three
HSCT for autoimmune disease in developing countries

The first patient with an autoimmune disease transplanted with hematopoietic stem cells in China was a lupus patient treated in 1998 at the Affiliated Drum Tower Hospital of the Nanjing University Medical College. After a visit by Dr. Richard Burt at Nanjing in 1999, three other centers launched their programs of HSCT for AID: the Union Hospital in Beijing, the Third People's Hospital in Zhengzhou and the Taian People's Hospital in Shandong.

The initial experience of the Nanjing University was reported in 1999 at the Worcester Translational Research Conference and subsequently in Chinese. Autologous bone marrow was collected from three SLE patients, cryopreserved at 4°C and infused after 56 h and conditioning with Cytoxan (CY) 120 mg/kg and Melphalan (140 mg/m²). G-CSF was given after transplantation and the three patients achieved clinical and laboratory remission. Subsequently, conditioning regimen was changed to CY (200 mg/kg) plus ATG (90 mg/kg) for SLE and BEAM + ATG for MS, and CD34+ selected and cryopreserved PBSC were used as the source of SC. The group at the Nanjing University performed HSCT for 18 patients with AID (seven SLE and 11 MS). Most patients improved after transplantation (5/7 SLE and 7/11 MS), one patient with SLE and three with MS showed stabilization of the disease, one MS patient progressed and another with lupus died of uncontrolled pulmonary hypertension and congestive heart failure 6 months post-transplantation. The Nanjing group intends to expand its series of autologous HSCT for SLE and MS, and target other inflammatory diseases such as rheumatoid arthritis, SSc, inflammatory bowel disease and myasthenia gravis. There are also plans of participation in phase III trials active in developed countries.

During the EBMT meeting in 2002, the Zhengzhou group reported its experience in autologous HSCT transplantation of 18 patients with SLE. Six patients received BM (median of 1.1 x 10^6 nucleated cells/kg) and 12 patients received PBSC (3.01 x 10^6 CD34+ cells/kg), neither product was manipulated in vitro. Mobilization was accomplished with CY (2 g/m²) plus G-CSF (250 µg/day) and the conditioning regimen combined total lymphoid irradiation (0-12 Gy), CY (50 mg/kg/day x 3) and rabbit ATG (Fresenius, 10 mg/kg/day at days +1 and +2) for in vivo T-cell depletion. Haematopoietic engraftment was achieved in 100% of patients with a median time of 15 days (12-18). B lymphocytes were markedly reduced and NK cells were increased 8-12 months post-transplantation. Two-thirds of the patients (12/18) had complete remission, three patients had a partial response and three patients did not respond after a median follow-up of 12 months (3-26 months), and there were no transplant-related deaths.

The Beijing group performed CD34-selected autologous HSCT in 11 patients (eight SLE, one RA, one SSc, one SJS). Conditioning was Cy + ATG (N = 7) or Cy + TBI (N = 4). All patients achieved clinical remission and three patients developed CMV infection. The Shandong group apparently did only one HSCT for SLE and the patient relapsed after transplantation.

In conclusions

Developing countries are able to produce significant results using HSCT for AID. In fact, the Chinese experience with lupus is already impressive regarding the numbers and outcome and the Brazilian experience is growing. Engagement in phase III randomized trials needs careful selection to answer specific questions relevant for our conditions such as the role of expensive in vitro manipulation of stem cells and the comparison of HSCT with expensive medical treatments such as anti-TNF agents for rheumatoid arthritis. Development of those trials depends on the results of present pilot studies, encouraging referral of early-stage patients, and availability of resources. Alternatively, we may join phase III clinical trials active in developed countries, but we may need their help for some resources. Finally, in our countries, one-shot therapy like HSCT is usually cheaper and have a more favorable cost-benefit ratio than prolonged immunosuppression needed for a subgroup of severely affected AID patients. Thus, we can benefit a large number of patients and give a significant contribution to the field, like that shown by Fassas et al in Greece transplanting MS. Our experience certainly will encourage other developing countries to overcome various obstacles and implement programs of HSCT for AID.

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The importance of lowering the costs of stem cell transplantation in developing countries.

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The effectiveness and efficiency of health care systems can be assessed by economic evaluation, comparing the costs and outcomes of alternative interventions. Direct costs include accommodation, nursing, physicians' fees, diagnostic tests and treatment. Indirect costs derive from the loss of a person's ability to use life in a productive way e.g. employment. In cost-effectiveness analyses, cost is the numerator and effectiveness (related to health outcomes) is the denominator. The unit of measurement of effectiveness is usually years of life saved. Cost utility analyses require a preference-based measurement of health-related quality of life (HRQL) to allow the calculation of utility scores for health states and so the adjustment of effectiveness for quality. Typically, this allows the calculation of quality-adjusted life years (QALYs). A review of published reports, to be presented in detail, yields the following summation. The greater cost of stem cell harvest from peripheral blood is more than offset by the reduced costs associated with a shorter hospital stay. Transplants early in first remission cost less than those undertaken at later points in disease evolution/treatment experience. Changing the primary locus of care from inpatient to outpatient may result in notable cost savings but can produce cost-shifting (from inpatient to outpatient). Nevertheless, in selected patient, the use of non-myeloablative transplants may offer a cost-effective option, especially in the developing country context.

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Bone marrow transplant cure for β-thalassaemia major: initial experience from a developing country

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Abstract Between July 2001 and June 2007, 48 consecutive patients with β-thalassaemia major received allogeneic haematopoietic stem cell transplants (allo HSCT) from human-leukocyte-antigen-matched siblings at the Armed Forces Bone Marrow Transplant Centre, Rawalpindi, Pakistan, using standard conditioning regimens. The median age of the patient cohort was 4 years (range, 1–14 years). Thirty-one patients were in risk class I, 11 in class II and six patients were in class III. Grafting was achieved in all patients. Survival was calculated from the date of transplant to death or last follow-up. Major post-transplant complications encountered were acute graft versus host disease (Ac GvHD) (grades II–IV), 35.4%; chronic GvHD, 8.3%; haemorrhagic cystitis, 12.5%; teno-occlusive disease (VOD) of the liver, 6.2%; bacterial infections, 37.5%; fungal infections, 19%; cytomegalovirus (CMV) infection, 6.2%; herpes infection, 6.2%; and tuberculosis in 2% of patients. Graft rejection was observed in five patients. Three patients received second transplants. Mortality was observed in 20.8% of patients. Major fatal complications included GvHD, VOD, intracranial haemorrhage, sepsicaemia, CMV disease and disseminated tuberculosis. Overall survival and disease-free survival were 79% and 75%, respectively, at 6 years post-HSCT.

Keywords Allo HSCT · β-Thalassaemia · Complications · Survival

Introduction

Thalassaemia probably represents the most common single gene disorder causing a major public health problem in the world. Thalassaemias are widely distributed in the Mediterranean, Middle Eastern and Asian countries and occur with significant incidence in populations that originate from these areas. In the Mediterranean and Arab gulf areas, there are more than 200,000 homozygous β-thalassaemia gene and homozygous birth rate is between 1:150 and 1:200. β-Thalassaemia is one of the major health problems in Pakistan. It is the most common genetic disorder in the country, with 5–8% gene frequency in the general population, and approximately 5,000 children are diagnosed each year in all ethnic groups [1–3].

Since the first successful case report of bone marrow transplant (BMT) in a child with β-thalassaemia in 1982, there has been ample evidence to show that BMT for clinically significant haemoglobinopathies can establish donor erythropoiesis and eliminate the underlying hereditary anaemia [4]. Bone marrow remains the predominant source of stem cells in allogeneic haematopoietic stem cell transplantation (HSCT) for non-malignant disorder like β-thalassaemia. During the past three decades, bone marrow transplantation has become a well-established treatment for β-thalassaemia [5, 6]. As per European Group for Blood and Marrow Transplantation registry 2312, thalassaemic patients received match-related donor transplants in 112 different centres by April 2004, and in 90% of transplant, bone marrow was used as stem cell source. Over the last 20 years, the Pesaro
Immune reconstitution and implications for immunotherapy following haematopoietic stem cell transplantation

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Abstract

Recovery of a fully functional immune system is a slow and often incomplete process following allogeneic stem cell transplantation. While innate immunity reconstitutes quickly, adaptive B- and especially T-cell lymphopoiesis may be compromised for years following transplantation. In large part, these immune system deficits are due to the decrease, or even absence, of thymopoiesis following transplantation. Therefore, T cell reconstitution initially relies upon expansion of mature donor T cells; a proliferation driven by high cytokine levels and the presence of allo-reactive antigens. This peripheral mechanism of T-cell generation may have important clinical consequences. By expanding tumouricidal T cells, it may provide a venue to enhance T-cellular immunotherapy following transplantation. Alternatively, decreased thymic function may impair long-term anti-tumour immunity and increase the likelihood of graft-versus-host disease.

Keywords

immune reconstitution; cellular immunotherapy; allogeneic stem cell transplant; thymus

INNATE IMMUNE RECONSTITUTION: NATURAL KILLER, NEUTROPHIL AND DENDRITIC CELLS

The innate immune system is comprised of a collection of cells that recognize and eradicate pathogens or aberrant cells without priming or antigen presentation. Natural killer (NK) cells, neutrophils, monocytes, dendritic cells (DCs) and macrophages contribute to innate immunity. NK cells purge tumour or virus-infected cells. By 1 month post transplant, NK cells circulate at normal levels and confer some degree of immune protection (Figure 1).1-6 These donor-derived NK cell clones effectively lyse recipient leukaemia in vitro.7 Studies have also shown that the number of NK cells early after transplant correlate with remission rates, implicating the function of these early cells in the clearance of residual tumour.8-10 Data also suggest that killer immunoglobulin-like receptor (KIR) mismatch may play a critical role in NK-cell-mediated tumour eradication.11,12 Many, but not all, studies have associated NK cell KIR mismatch with protection from relapse and graft-versus-host disease (GVHD) in human leukocyte antigen haplotype mismatched and matched unrelated transplants for myeloid and acute lymphoid malignancies.5,11,12

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CONFLICT OF INTEREST STATEMENT

None declared.
Like NK cells, recovery of neutrophils and monocytes is rapid following transplantation; however, the timeline for DC reconstitution is delayed and falls between that of innate and adaptive immune recovery. DCs process and present antigens to the adaptive immune system, while producing inflammatory cytokines important for stimulation of the innate immune system. After stem cell transplantation, although donor DCs can be detected in the peripheral blood within the first few weeks, the total number may not approach normal for more than 1 year in adults (Figure 1). Furthermore, studies suggest that while the peripheral blood DCs are largely of donor origin (>80% by Day 14), up to 70% of tissue DCs may remain of host origin after SCT. These host origin tissue DCs may persist for up to 1 year following SCT. Furthermore, the functional competency of these newly derived DCs is not yet well understood in the context of stem cell transplantation. As DC-loaded peptides engender better infectious responses than vaccine alone following transplantation, there is evidence that DC function may be impaired post-transplant, although these studies are limited by differing diseases and transplant conditions.

Collectively, these data suggest that many of the components of the innate immune system reconstitute relatively quickly and completely in the early post-transplant period. While this quick, functional recovery of innate immunity may be exploited for tumor immunotherapy, to date, few studies have demonstrated that these innate immune cells can be manipulated to maximize graft-versus-leukaemia (GVL) effects, with the one notable exception of acute myeloid leukaemia (AML). Some studies have suggested that a graft with KIR mismatch may predict a high probability of NK alloreactivity, augment the graft-versus-AML effect, and diminish the risk of relapse following transplantation. Unfortunately, for most patients and diseases, the data for this NK-alloreactive effect are limited. Additionally, because little is known about the kinetics and function of tissue DCs following transplantation, mechanisms to exploit this biology in vivo have yet to be elucidated. Indeed, the few studies that have investigated anti-tumour vaccination strategies after transplantation have included the administration of DCs loaded with specific peptides to obviate the need for functional donor DCs in vivo (see below).

**ADAPTIVE IMMUNE RECONSTITUTION: B AND T CELLS**

In contrast to innate immune cells, the lymphoid reconstitution of the adaptive compartment is delayed, resulting in persistent recognized deficits in terms of global immunity. In large part, deficiencies in T- and B-cell function after transplant are due to impaired thymopoiesis. As a result, T-cell reconstitution relies upon an alternate pathway of development, termed 'homeostatic peripheral expansion' (HPE). In HPE, mature donor T cells expand, rendering a limited repertoire of T cells available to recognize and signal B-cell counterparts. B and T cells require priming by antigen and engender long-term specific responses using unique receptor sequences. In non-transplant subjects, as B and T cells have the potential for long-term and specific immunity, they are attractive targets for cellular immunotherapy. However, the protracted timeline for lymphoid recovery after transplant remains a challenge to this approach following transplantation. Circulating B cells may not reach normal levels for at least 12 months post transplant and T-cell reconstitution may be delayed for more than 2 years. Despite this fact, there are aspects of the immediate post-transplant milieu that may be especially conducive to anti-tumour T-cell responses, whereas high levels of cytokine that can fuel cytotoxic T-cell proliferation and activation. Thus, an understanding of the cytokine- and antigen-driven responses that are possible following transplantation may permit the genesis of a platform for anti-tumour treatments, with cautious consideration to the potential for exacerbation of GvHD.
B-CELL IMMUNE RECONSTITUTION

B-cell development

B cells are lymphocytes generated in the bone marrow from common lymphoid progenitors. B cells possess immunoglobulin receptors that are generated by somatic recombination, whereby genes are re-arranged to create diverse receptor sequences. B cells undergo selection in the bone marrow prior to release into the peripheral blood. Naïve mature B cells emerge from the marrow with surface immunoglobulin (Ig) M and IgD receptors, then migrate to secondary lymphoid structures. Once a B cell encounters an antigen, often through interactions with CD4+ T cells or DCs, it becomes activated and releases IgM. The activated B cell then processes the antigen for presentation to T cells. After T-cell stimulation, the B cell may undergo isotype switching such that it expresses IgG, IgA or IgE on the cell surface. The B-cell receptor/immunoglobulin molecule may then undergo further receptor modification in the variable regions (VH), through somatic hypermutation, increasing the avidity of the antibody/epitope interaction.

B-cell development

Following allogeneic stem cell transplantation, B-cell development recapitulates that of normal ontogeny in terms of circulating cell types and numbers. In normal ontogeny, B-cell numbers rise until the toddler years, after which there is a gradual descent to normal adult levels in the peripheral blood. Following stem cell transplantation, levels of circulating B cells are very low for the first few months, but then reach levels exceeding normal adults by 1-2 years, approaching the range of normal neonates (Figure 1). 32 The levels then gradually fall. Post-transplant, donor B cells emerge with a naïve phenotype (IgM+ IgD+) initially, with memory B-cell development up to 5 years later.33,35 Consistent with this, IgM levels recover first, by 2-6 months,36 followed by IgG levels which approach normal between 3 and 18 months following transplantation. Finally, IgA reconstitution may be delayed for up to 3 years.6,37 It is important to note that the IgG levels may, in part, reflect residual host plasma cell production, and thus may not be entirely suggestive of donor B-cell competence because host plasma cells are relatively resistant to current preparative regimens and may persist for up to 2 years.38,39

B-cell function following transplantation

The function of B cells remains compromised for 1-2 years following stem cell transplantation. In part, this has been attributed to the T-cell defects during this time frame, with maturation arrest at the naïve stage due to insufficient CD4+ T-cell signalling. After transplant, memory B cells that require CD4+ help for isotype switching show a skewed pattern of complementary determining regions 3 (CD3) of the immunoglobulin heavy chain.40,41 In addition to the lack of CD4+ help, there appears to be an environmental defect following transplantation, because the rate of somatic hypermutation is decreased in mature B cells even in the presence of normal donor CD4+ T cells.42 Thus, following transplantation, B-cell immunity is impaired due to: (1) prolonged low levels of circulating B-cell numbers; (2) a relative defect of mature B cells secondary to decreased isotype switching; and (3) a diminished ability to undergo somatic hypermutation. Importantly, this decreased B-cell function following stem cell transplantation has resulted in diminished vaccine responses to infectious antigens, even after normal B-cell numbers have been achieved.26,43,44

T-CELL RECONSTITUTION

Thymic-dependent T-cell reconstitution

The thymus is the primary site for T-cell development. When peripheral T-cell populations are severely depleted, renewed thymic activity can contribute to T-cell reconstitution, producing naïve CD4 helper, CD8 cytotoxic effector and CD4+ CD25+ regulatory T cells. During
thymopoiesis, bone-marrow-derived T progenitors migrate to the thymus, expand and mature. Developing thymocytes acquire a T-cell receptor (TCR), generated through recombinant real-arrangement of variable (V), diversity (D) and joining (J) genes. The re-arrangement of these genes within the thymus ensures TCR diversity. Prior to emigration as naive T cells, these TCRs undergo positive and negative selection, deleting autoreactive clones termed 'central
tolerance'.

Renewed thymopoiesis may be assessed by several techniques including thymic imaging, naive T-cell frequency, TCR excision circle quantity, and spectrotopy pattern. With renewal of thymopoiesis, the proportion of cells with a 'naive' phenotype increases in peripheral T-cell populations. Measurement of TCR re-arrangement excision circles (TRECs) provides a means of quantifying thymic productivity. TRECs are episomal DNA circles that are generated as a byproduct from the spliced DNA elements remaining after re-arrangement of the VDJ genes encoding the TCR α and β chains. TRECs are not replicated during cell division but are retained in the thymic emigrant cell, and thus are diluted with cell division. Robust thymopoiesis will be reflected in high frequencies of TREC-bearing cells, termed 'recent
thymic emigrants'. In contrast, with intense peripheral expansion, peripheral TREC frequencies remain low. Finally, thymic function may also be evaluated by spectrotyping, a polymerase chain-reaction-based analysis of length variation in the CDR3 of the TCR β chain. A productive thymus is reflected in a Gaussian distribution of CDR3 lengths because of the enormous diversity generated by VDJ re-arrangement occurring in maturing thymocytes. In contrast, clonal expansion results in an oligoclonal pattern of limited CDR3 lengths. Renewal of thymopoiesis thus re-establishes polyclonal Gaussian patterns in the CDR3 spectrotypes, first in naive cells and subsequently in memory T cells.

T-cell reconstitution by homeostatic peripheral expansion

An alternate pathway for T-cell development is through the rapid cell division of mature T-cell clones, termed 'HPE'. This was first demonstrated in a murine model in which two very distinct patterns of T-cell reconstitution ensued following the adoptive transfer of syngeneic bone marrow cells and congenic lymph node cells into C57BL/6-irradiated, thymus-intact or thymecomized recipients. Thymus-bearing hosts largely reconstituted with syngeneic marrow-derived T cells through a thymus-dependent mechanism. Thymectomized mice derived the majority of peripheral T cells from the congenic lymph node innocula, via HPE. HPE drives T-cell reconstitution in lymphopenic hosts and is associated with a shift from naive to memory/activated phenotype in the proliferating cells.

HPE is dependent upon the support of homeostatic cytokines and cognate antigen-driven and regulatory cellular interactions. Interleukin (IL)-7 and IL-15 are the cytokines that drive HPE of naive and CD8+ memory T cells, respectively. Regulatory cells and tumour growth factor beta (TGFβ) provide cellular constraints on HPE. Finally, antigen presentation can drive HPE in lymphopenic and transplant recipients.

IL-7 is a critical, non-redundant cytokine required for stimulating naive T-cell expansion and sustaining naive T-cell survival. Human and animal studies have shown that IL-7 markedly increases naive CD4+ and CD8+ T cells, diminishing TRECs frequency. Conversely, IL-7 depletion restricts T-cell expansion through cytokine depletion by a consumption-based mechanism. Current data suggest that lymphocyte depletion following preparative regimens leads to an increase in cytokine availability (presumably due to lack of cells consuming the cytokine). Upon infusion of donor T cells, the surplus IL-7 drives native cells into proliferation until T-cell numbers return to a level at which IL-7 is once more a limiting factor. Human studies initially revealed this inverse correlation between serum levels of IL-7 and T-cell reconstitution after lymphodepletion; high serum levels of IL-7 coincided with severe
lymphopenia, which decreased rapidly with lymphocyte recovery following transplantation. 53,54

Recent evidence suggests that IL-15 as well as IL-7 may act as a homeostatic cytokine, supporting HPE in lymphopenic hosts. IL-15 enhances proliferation of human CD8+ memory populations in vitro. 55,56 It is produced constitutively by tissues and antigen-presenting cells, and is upregulated in the setting of inflammation. When lymphocytes have been severely depleted as in the setting of bone marrow transplantation, plasma IL-15 levels increase dramatically and exceed normal levels concomitant with a disproportionate expansion of CD8+ memory cells. 57-60 Consistent with this finding, IL-15R expression and responsiveness are highest on activated memory CD8+ T cells. 61-63

Since IL-15 and IL-7 are produced constitutively and could lead to unchecked T-cell expansion, these are balanced by factors that negatively regulate T-cell subsets. TGFβ antagonizes the effect of IL-15, curtailing the proliferation and persistence of CD8 central memory populations. 64,65 Administration of TGFβ to human and murine CD8+ memory cells in vitro decreases CD8 proliferation and attenuates effector function. 66,67 Similarly, regulatory T cells (Tregs) constrain the HPE of naive CD4+ and CD8+ T cells, influencing the host reactivity of T cells during immune reconstitution. 68 Tregs are CD127-CD25+ CD4+ T cells that function to control auto-immune responses. 69,70 Characterized by the expression of the transcription factor Foxp3 and a high level of glucocorticoid-induced tumour necrosis factor receptor, Tregs have been shown to modulate the peripheral expansion of T cells to low- and high-affinity antigen in lymphopenic hosts. 68,70 Treg levels appear to be low following transplantation or chemotherapy, but expand rapidly in the first month. 71,72

In addition to cytokine and cellular regulation of HPE, antigen presentation can also direct the recovering T-cell compartment. First demonstrated in murine models, peripheral expansion of TCR transgenic cells was increased substantially in the presence of cognate antigen. 73 This mechanism was further characterized as a rapid, IL-7-independent expansion of CD4+ and CD8+ T cells that is dependent on major histocompatibility complex II interactions and CD28 ligation, 74 and termed "endogenous proliferation," to differentiate it from homeostatic proliferation which is driven by cytokine signals. 75 In irradiated lymphopenic hosts, both processes could be demonstrated for subsets of CD4+ and CD8+ cells during immune reconstitution. 76 In allogeneic transplantation, the influence of endogenous proliferation is best demonstrated by the disproportionate expansion of cytomegalovirus (CMV)-reactive CD8+ T cells observed in CMV-positive individuals. 76,77 Thus, although homeostatic proliferation permits the expansion of the T-cell compartment as a whole following stem cell transplantation, endogenous proliferation directs T cells to proliferate in response to specific antigen stimuli early in T-cell recovery.

T-cell reconstitution by peripheral expansion in the initial post-transplant period and implications for immunotherapy

In the early post-transplant period, elevated levels of IL-7 and IL-15 drive homeostatic expansion of residual (host) or infused (donor) T-cell subsets. Thymopoiesis is significantly impaired due to thymic injury from the preparative regimen, diminished thymic function in adults, the lack of circulating T-cell precursors, and the absence of critical cytokines or growth factors. This early reliance of the T-cell compartment on HPE has profound consequences. The relative frequencies of the T subsets shift towards a preponderance of activated and cycling memory T cells. 78 As T cells expand through HPE, the frequency of TREC declines. 79 T cells, especially CD8+ subsets, expand rapidly in the first weeks, but this expansion is often unstable with longitudinal studies describing a sharp decline in overall T-cell numbers by 3-6 months. 58 Robust CD8+ proliferation and impaired CD4+ reconstitution lead to an inverted
CD4:CD8 ratio in the months to years following stem cell transplantation. The TCR repertoire resulting from these early expansions is typically skewed and oligoclonal.

This oligoclonal peripheral expansion of relatively few donor T cells potentially presents an opportunity for cellular immunotherapy. Murine models have shown that severe lymphodepletion establishes optimal conditions to promote graft-versus-tumour due to the high levels of homeostatic cytokines (IL-7 and IL-15) and the depletion of regulatory T cells that impede immune responses. Higher doses of irradiation yielded superior anti-tumour responses when mice were given transgenic melanoma-antigen-specific T cells. Furthermore, this effect was abrogated in the absence of IL-7 and IL-15 when these knock-out mice were used as recipients. Depletion of regulatory T cells also enhanced the tumouricidal effect. In the setting of vaccination, while Tregs can also proliferate rapidly and hamper effective anti-tumour responses in lymphoreplete hosts, following stem cell transplantation, the Treg compartment is constrained (by IL-2) while anti-tumour effectors expand and out-compete Tregs. Murine stem cell transplantation models showed that the post-transplant period provides an optimal milieu for cellular immunotherapy, demonstrating specific anti-melanoma immunity and tumouricidal effect when either a vaccine and cytokine adjuvant, a tumour lysate DC pulsed vaccine, or GM-CSF-producing tumour vaccine were administered in the post-transplant lymphopenic setting.

Human studies have corroborated murine data, showing maximal anti-tumour T-cell expansion in the setting of severe lymphopenia. Studies in the highly immunogenic tumour melanoma have demonstrated the potential for this platform. Ex-vivo-expanded melanoma-infiltrating lymphocytes administered with IL-2 after cytoreductive therapy led to clinical responses in up to 50% of patients evaluated. Furthermore, a single study has even demonstrated that T cells can be genetically engineered to target melanoma and affect clinical responses when given during intense lymphopenia.

Vaccination strategies using peptide-pulsed DC vaccines for melanoma have also demonstrated T-cell-specific responses with some clinical responses. It should be noted that these DC-pulsed peptide vaccines did not incorporate lymphodepletion in the regimens. In haematological malignancies, this framework for vaccines has only recently been incorporated into clinical trials, with few studies reported to date. In contrast to melanoma, when peptide-pulsed vaccines were administered in the post-transplant setting (autologous or allogeneic) for multiple myeloma or AML, some assays could detect an immunological response but clinical responses were absent. Notably, these studies administered the vaccines months following transplantation, after exponential T-cell proliferation has likely occurred and IL-7 and IL-15 cytokine levels would be expected to be low. These studies suggest that peptide-pulsed vaccines may be used safely after stem cell transplantation, and that alternative timing strategies may actually lead to a clinical response. Given the successful strategies in melanoma, future trials may seek to incorporate infusion of tumour-targeted T cells and/or the administration of DC vaccines in the early post-transplant period to capitalize on the cytokine storm and absent Treg populations to potentiate tumouricidal effects.

**T-cell reconstitution by thymopoiesis following transplantation and implications for cellular immunotherapy**

While early post-transplant T-cell reconstitution relies upon HPE, an optimal T-cell armamentarium with a diverse TCR repertoire is only achieved through the (delayed) recovery of thymopoiesis (Figure 2). This may have important implications for cellular immunotherapy, particularly in adults who demonstrate limited residual thymic activity. Without renewal of thymopoiesis, naïve T cells and total CD4 + T-cell reconstitution is impaired indefinitely. In paediatric patients, CD4+ naïve T cells are delayed for 1 year or more until thymic activity has resumed. Even 20 years following transplantation,
While the CD8+ rich, memory, oligoclonal population of T cells due to HPE may be beneficial as a platform for vaccination therapy, the delay in thymopoiesis following transplantation may ultimately restrict vaccine efficacy. The lack of diversity of TCRs could diminish the likelihood of an appropriate TCR for tumour antigen recognition. Murine models have demonstrated that decreased TCR diversity due to age or thymectomy correlated with diminished response to an infectious antigen. Furthermore, since CD4+ T cells are necessary for longlasting B- and CD8+ T-cell immunity, the persistent defects in CD4+ T cells likely contribute to impaired responses to infectious and tumour vaccines following transplantation. Murine data suggest that optimal vaccination responses occur in the setting of active thymopoiesis. In human trials, impaired thymic function has been correlated with increased incidence of relapse, suggesting that renewed thymopoiesis may enhance anti-tumour immunity.

In addition to mitigating the effects of cellular immunotherapy, thymic defects are associated with increased risk of acute and chronic GvHD which further impeded de-novo immune responses. In a paediatric population, GvHD and low TREC levels indicative of poor thymic function even conferred an increased risk of mortality. Post-transplant thymic dysfunction contributes to the onset and persistence of GvHD. While thymic damage permits the escape of alloreactive T cells that may engender GvHD, the thymus is also a target of these allo-reactive T cells, limiting thymic renewal. Thus, the regeneration of thymic structures is stunted and denovo diverse T production is impaired indefinitely. Of critical importance when considering T-cell immunotherapy in the context of stem cell transplantation is the barrier that factors that support GvL may worsen GvHD. In murine studies, high doses of IL-7 and IL-15, through exogenous administration or transgenic models, increased the severity and frequency of GvHD. In human studies, persistence of high IL-15 levels following allogeneic stem cell transplantation was correlated with severe acute GvHD. In contrast, factors that reduce peripheral expansion can reduce GvHD. Regulatory T cells could control homeostatic expansion in lymphopenic mice and prevent acute GvHD in murine models. Similarly, in clinical trials, the level of FoxP3+ T regulatory cells in the donor inoculum was inversely correlated with development of acute GvHD.

STRATEGIES TO OPTIMIZE CELLULAR IMMUNOTHERAPY FOLLOWING TRANSPLANTATION

As T- and B-cell reconstitution is critical to successful immunotherapy approaches following transplantation, maximal anti-tumour responses are likely to be elicited when lymphoid reconstitution is both rapid and functional. In part, efforts should be directed towards enhancing thymic recovery while potentially directing the immediate post-transplant HPE towards tumour-specific antigens. Thymic function is influenced by: age, stem cell source; GvHD (see above); the degree of damage due to the preparative regimen; and possible cytokine, hormonal and growth factors. Host age affects thymic recovery significantly, with each successive decade reducing the rate and potential for thymic renewal. In many older adults, recovery of naïve cells through thymopoiesis occurs 3-5 years following transplantation; in some, naïve cells remain below normal levels for decades after stem cell transplantation. While host age is an immutable challenge to thymic recovery, other factors may be influenced by transplant design. To reduce thymic damage, less intensive preparative regimens have enhanced CD4+ T-cell reconstitution and T-cell repertoire diversity, although these may also reduce the levels of HPE cytokines available for early T-cell expansion. Increasing the availability of functional T-cell progenitors has enhanced thymopoiesis in murine models. In human studies, umbilical cord stem cells resulted in higher TREC.

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frequencies than adult bone marrow stem cells, possibly due to a higher frequency of T-cell progenitors in these marrows. Furthermore, manipulation of thymic cytokines, growth factors or hormones has improved thymic function after stem cell transplantation. As IL-7 is an important survival factor for developing thymocytes, administration of this cytokine in murine transplant models has enhanced thymopoiesis following bone marrow transplantation. Similarly, keratinocyte growth factor (KGF) has shown promise in boosting thymic productivity in murine hosts. Human studies utilizing KGF have not confirmed these data; however, they were not designed to evaluate enhanced thymopoiesis. Finally, androgen withdrawal and growth hormone have been associated with enhanced thymopoiesis in murine and human studies.

In addition to the factors that affect thymic recovery, the transplant design may enhance immunotherapy by targeting the initial expansion of allo-reactive T cells in the graft. Stem cell source has been studied separately and shown to influence HPE-derived early T-cell reconstitution. Peripheral blood stem cell grafts initially reconstitute peripheral T-cell numbers more rapidly. T-cell depletion and GvHD have also been shown to delay T-cell reconstitution. Degree of mismatch may also influence early T-cell recovery; partially matched or unrelated donors demonstrate delayed T-cell reconstitution with skewed repertoires and very low TREC frequency.

**SUMMARY**

Immune reconstitution following stem cell transplantation does not recapitulate that of ontogeny. This has important implications for the development and maintenance of tumour immunotherapy. While most of the innate immune system recovers quickly and completely, lymphoid reconstitution may be quite delayed in terms of cell number and global function. In large part, lymphoid recovery is hindered by the lack of thymopoiesis. As a result, T-cell reconstitution relies upon an alternate pathway of development that expands infused or residual cells in response to cytokines and antigens. Furthermore, donor DC renewal may also occur late after transplant, minimizing the capacity for these cells to present tumour antigens effectively and alert the developing immune system to the presence of minimal residual disease. These delays in lymphoid and DC reconstitution have significant clinical ramifications, permitting the exploitation of the initial T-cell expansion while challenging effective long-term immunity in the absence of thymopoiesis, appropriate B-cell signalling (due to the absence of CD4+ T cells) and functional donor DCs.

However, these components of immune reconstitution may be manipulated to aid GvL effects. Optimal stem cell transplantation involves eradication of residual tumour, long-term anti-tumour immunity, and minimal GvHD. For rare patient and donor pairs, the early functional recovery of NK cells affords considerable anti-tumour benefit. However, for most graft-host pairs, the optimal transplant entails achieving a balance between the peripheral expansion of anti-tumour T cells and thymic reconstitution, rendering a continuous infusion of de-novo T cells for tumour immunosurveillance with a diverse repertoire to minimize the likelihood of severe GvHD. To maximize stem cell transplantation for immunotherapy, future studies may alter the donor graft to direct the initial homeostatic T-cell expansion towards tumour eradication, either by infusing genetically engineered anti-tumour T cells, expanding naturally occurring GvL T cells prior to infusion, and/or administering DC vaccines loaded with peptides to drive a specific anti-tumour T-cell response. For the best effect, current immunological data suggest that these therapies should be given in the immediate post-transplant period to capitalize on the surfeit of cytokine levels. Alternatively, one could consider administering IL-7 or IL-15 after the first month to raise the levels and drive HPE of anti-tumour T cells after the cytokine surplus is depleted, although this should be considered cautiously in allo-transplantation because of the heightened risk of GvHD. While CD8+ T cells are typically
targeted for these therapies, treatments that employ CD4+ T cells may lead to longer lasting immunity and permit a more effective B-cell response. In the initial period, administration of CD4+ T cells may help to achieve B-cell responses, although the greatest immunotherapy benefit is likely to be obtained when thymopoiesis is augmented as well, replenishing naïve CD4+ T cells. Hopefully, future studies will advance thymic renewal while directing the initial homeostatic T-cell expansion towards tumour eradication. In this way, both pathways of T-cell development can be maximally exploited towards improved GvL immunotherapy without stimulating GvHD, leading to long-term fully functional lymphoid immunity and successful tumour and infection clearance.

**Practice points**

- Single immunity, including neutrophils and NK cells, recovers quickly and completely.
- Donor DC competence is delayed.
- T-celland, to some extent, B-cell immune reconstitution is delayed following stem cell transplantation.
- The initial post-transplant period is dominated by cytokine-driven peripheral expansion of mature donor T-cells.
- Recovery of thymopoiesis is impaired after stem cell transplantation and contributes to T- and B-cell incompetence.

**Research agenda**

- The initial post-transplant period may provide a platform for successful T-cell immunotherapy strategies, due to the robust peripheral expansion during this time frame.
- Strategies to aid in thymic renewal may improve long-term tumour immunosurveillance.

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Figure 1.
Representative time lines of immune reconstitution in adult patients without extensive graft-versus-host disease, infections or relapse. NK, natural killer; DC, dendritic cells. 1,2,4,18, 20-22,31,103,105
Figure 2.
Diagrammatic representation of the clinical consequences of the two types of T-cell reconstitution evident in the post-transplant period. Early after stem cell infusion, homeostatic proliferation drives the T-cell repertoire due to the high levels of cytokines and delayed thymic recovery. As a result, T cells in the donor product expand and mature into the memory pool, leading to an expanded memory pool with decreased diversity in both naïve and memory lymphocytes. Given the rapid proliferation of few T cells, this immediate post-transplant period presents an opportunity for immunotherapy, through the administration of tumour-targeted T cells or through vaccine administration. Later, in patients with the potential for thymic recovery (including younger patients without graft-versus-host disease (GvHD)), renewed thymopoiesis enriches the naïve T-cell pool. Due to the T-cell receptor re-arrangement in the thymus, these recent thymic emigrants enhance the diversity of the naïve, and subsequently the memory, T-cell repertoire (diagrammatically shown as multi-patterned cells). This has significant implications for long-term immunity. Not only are these patients less prone to life-threatening infections and GvHD, the enhanced T-cell diversity and renewed CD4+ cells likely confer improved tumour immunosurveillance, as shown by decreased rates of tumour relapse. HPS, homeostatic peripheral expansion; TREC, T-cell receptor re-arrangement excision circle, IL, interleukin, Treg, regulatory T cells.
Haploidentical stem cell transplantation after a reduced-intensity conditioning regimen for the treatment of advanced hematologic malignancies: posttransplantation CD8-depleted donor lymphocyte infusions contribute to improve T-cell recovery

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Haploidentical hematopoietic stem cell transplantation provides an option for patients with advanced hematologic malignancies lacking a compatible donor. In this prospective phase 1/2 trial, we evaluated the role of reduced-intensity conditioning (RIC) followed by early add-backs of CD8-depleted donor lymphocyte infusions (DLIs). The RIC regimen consisted of thiotepa, fludarabine, cyclophosphamide, and 2 Gy total body irradiation. Twenty-eight patients with advanced lymphoproliferative diseases (n = 24) or acute myeloid leukemia (n = 4) were enrolled. Ex vivo and in vivo T-cell depletion was carried out by CD34⁺ cell selection and alemtuzumab treatment. The 2-year cumulative incidence of nonrelapse mortality was 26% and the 2-year overall survival (OS) was 44%, with a better outcome for patients with chemosensitive disease (OS, 75%). Overall, 54 CD8-depleted DLIs were administered to 23 patients (82%) at 3 different dose levels without loss of engraftment or acute toxicities. Overall, 6 of 23 patients (26%) developed grade II-IV graft-versus-host disease, mainly at dose level 2. In conclusion, our RIC regimen allowed a stable engraftment with a rather low nonrelapse mortality in poor-risk patients; OS is encouraging with some long-term remissions in lymphoid malignancies. CD8-depleted DLIs are feasible and promote the immune reconstitution. (Blood. 2009; 113:4771-4779)

Introduction

Allogeneic stem cell transplantation (SCT) is a curative option for several hematologic malignancies. However, its application is frequently limited by donor availability. Other sources of stem cells include matched unrelated donors, cord blood units, and haploidentical family donors. Haploidentical SCT was unsuccessful for many years because of graft rejection and high incidence of acute graft-versus-host disease (GVHD). The use of megadose of highly purified CD34⁺, pioneered by the Perugia group, allowed a high engraftment rate with a limited incidence of acute GVHD.¹,² Sustained engraftment and rapid natural killer (NK)-cell reconstitution were also achieved using CD3/CD19-depleted grafts.³

In adults, the relevant nonrelapse mortality (NRM) and disease recurrence remain the major obstacles to the routine use of haploidentical SCT. NRM is mainly caused by infections resulting from delayed immune reconstitution in the first 12 months after SCT. Disease recurrence may be partly explained by the use of extensive T-cell depletion, which impairs the graft-versus-tumor effect.

Donor-versus-recipient NK alloreactivity has emerged as a crucial factor for the outcome of haploidentical SCT. Ruggeri et al showed a low relapse risk for patients with acute myeloid leukemia transplanted from NK-alloreactive donors.⁴ Because the chance of finding an alloreactive donor is approximately 40% to 50%, new strategies are required for patients lacking an alloreactive donor or affected by hematologic malignancies that are resistant to NK-cell cytotoxic activity.

Adoptive immunotherapy, using unmanipulated donor T cells from haploidentical family donors, is probably not feasible because of the extremely high risk of severe acute GVHD. Infections of donor T cells, depleted of alloreactive lymphocytes by anti-CD25 immunotoxin or transduced with suicide genes, have been recently investigated to improve immune reconstitution.⁵,⁶

In human leukocyte antigen (HLA)-matched sibling transplantations, CD8 depletion of donor lymphocytes was used to reduce the incidence and severity of acute GVHD while preserving the anti tumor response.⁷,⁸ Nevertheless, contamination by residual CD8⁺ T cells was too high to allow its use in unrelated or haploidentical settings. In 2007, Meyer et al administered CD8-depleted donor lymphocyte infusions (DLIs) after unrelated SCT with an 18% incidence of GVHD. They obtained a very limited CD8⁺ T-cell contamination using the Miltenyi ClinMACS device (Miltenyi Biotech, Auburn, CA).⁹ In addition, Zheng et al have shown that CD4⁺ effector memory T cells could be added safely to T-cell-depleted major histocompatibility complex-mismatched allograft in the murine model.¹⁰ These cells promoted both graft-versus-leukemia and immune reconstitution with far less toxicity.


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GVHD than would be expected by unfractionated T cells or by naive CD4+ T cells.11

Here, we report the results of a phase 1/2 prospective trial in which haploidentical SCT using a reduced-intensity conditioning (RIC) regimen was followed by early add-backs of CD3-depleted DLIs.

Methods

Patient characteristics

Between January 2003 and January 2007, we conducted a prospective phase 1/2 trial. Twenty-eight patients received a transplantation from haploidentical family donors. Patients with advanced relapsed or refractory hematologic malignancies were eligible if they fulfilled the following criteria: (1) absence of HLA-related or unrelated donors and (2) a failed autologous SCT and a transplantation compatibility score more than or equal to 2. Nineteen patients younger than 50 years came from a failed autologous SCT (n = 17) or high-dose chemotherapy (n = 2). Eleven had a SCT compatibility score more than or equal to 2 according to SorREN et al.12

Family members were assessed for HLA compatibility by high-resolution molecular analysis (HLA-A,-B,-C,-DR,-DQ). Patients were screened for detection of anti-HLA antibodies by multiplex Luminex (Biosource International, Camarillo, CA) and excluded in case of positivity. Potential donor-versus-recipient NK-cell alloreactivity was analyzed according to missing expression of HLA-C groups 1 or 2 and of HLA-Bw4 alleles. The protocol was approved by the Institutional Review Board and Ethics Committee of the Fondazione Istituto di Rienner e Curara Carattere Scientifico Istituto Nazionale dei Tumori. All patients gave their written informed consent to participate in accordance with the Declaration of Helsinki. Patient and disease characteristics are summarized in Table 1. Median age was 38 years (range, 15-65 years). Only 13 patients were chemosensitive at the time of transplantation (n = 8 complete remission, n = 5 partial remission). The median time from diagnosis to transplantation was 29 months (range, 7-130 months). Twenty-one of 28 patients (75%) came from a failed autologous SCT. Twenty-three patients (83%) were at risk for cytomegalovirus (CMV) reactivation (CMV-seropositive recipient (R CMV+ receiving graft from CMV-seroconjugous [D CMV+] or -seronegative donors [D CMV−]).

Conditioning regimen, stem cell mobilization, and supportive care

The preparative regimen included thiopeta (10 mg/kg) on day −7; cyclophosphamide (30 mg/kg) on days −6 and −5; and fludarabine (30 mg/m2) from day −6 to day −3, aldesleukin (15 mg/m2) on day −2, and total body irradiation (2 Gy) on day −1. Donors received eflizumab 8 μg/kg twice daily beginning 4 days before leukapheresis. CD34+ cells were selected using the CliniMACS device (Miltenyi Biotec). The target doses of CD34+ and CD19+ T cells were 8 × 10^6/kg and 10^6/kg, respectively. No immunosuppressive therapy or posttransplantation granulocyte colony-stimulating factors were given. Patients were managed in laminar airflow rooms. Red cell and platelet transfusions were given according to institutional policy. Prophylaxis against viral and fungal infections consisted of high-dose acyclovir (500 mg/m2 every 8 hours daily) and liposomal amphotericin-B (1 mg/kg daily) from day −7 until the end of neutropenia. Prophylaxis after neutrophil recovery consisted of acyclovir, flucytosine, sulfamethoxazole, and trimethoprim-sulfamethoxazole until recovery of CD4+ cells. Blood samples from patients were screened weekly for CMV and Epstein-Barr virus (EBV).
CD8-depleted DLIs

Donors collected lymphocytes 2 weeks before allograft mobilization. The depletion of CD8+ T cells was performed using ClinMACS CD5 Microbeads and the ClinMACS CD8+ device (Miltenyi Biotec) with a good manufacturing practice procedure. Patients received CD8-depleted DLIs after SCT if they had no signs of active GVHD or rapidly progressive disease. Dose level 1 consisted of 10-fold CD8-depleted T cells on days +45, +75, and +105. If one case of acute GVHD more than 2 occurred in this cohort, the DLI program would have been stopped. Dose level 2 consisted of 5 × 106/kg CD8-depleted T cells on days +45, +75, and +105. Dose reduction was required if 2 cases of acute GVHD more than 2 occurred at this level.

Chimerism analysis and immunophenotype analysis

Chimerism was assessed on DNA extracted from peripheral blood samples by multiplex short-tandem repeat analysis (AmpliTSTR Profiler Plus PCR kit; Applied Biosystems, Foster City, CA). Peripheral blood was collected in ethylenediaminetetraacetic acid for lymphocyte analysis at 1, 3, 4, 5, 6, 9, 12, and 18 months after haploidentical SCT. Direct 2- or 3-color flow cytometric immunophenotyping was performed to analyze lymphocyte subsets, including CD3+, CD19+, CD14+, CD56-1, CD3-CD19+, and CD3-CD16/CD56+. CD3+CD14+ cells were scored. CD14+ monocytes were identified from the CD14+CD106+ population and were not part of the T cell population. CD3-CD56+ cells were identified from the CD56-1 population. Samples were analyzed using the FACSComp flow cytometer (BD Biosciences). Assays were not performed if the T cell dose was less than 106/kg.

Endpoints of the study and statistical analysis

The primary endpoints of the study were engraftment and NRM at 1 year. The secondary endpoints were the incidence of grade II-V acute GVHD before and after CD8-depleted DLIs; GVHD was assessed by consensus criteria.16,17 Overall survival (OS) and progression-free survival curves were estimated by the Kaplan-Meier method and compared using the log-rank test. Crude cumulative incidence curves of relapse and NRM were estimated in a competing risk framework.18 Comparative analysis between dose level 1 and dose level 3 was performed with Mann-Whitney test.

Results

Engraftment and chimerism

The stem cell target dose was achieved with a median of 2 leukaphereses (range, 1-3). The median number of CD34+ and CD3+ cells infused was 10.5 × 106/kg (range, 1.5-15 × 106/kg) and 1 × 106/kg (range, 0.5-5 × 106/kg), respectively. Median CD3+ purity, using the Miltenyi Biotech device, was 95% (range, 81%-98%). The median number of CD19+ and CD56+ cells was 9.5 × 106/kg (range, 1.70-10 × 106/kg) and 1.1 × 106/kg (range, 0.23-10 × 106/kg), respectively. One donor was a poor mobilizer (final inoculum contained only 1.5 × 106/kg CD34+); the donor received a combination of peripheral blood and bone marrow cells achieving long-term engraftment. Potential donor-versus-recipient NK alloreactivity was detected in 11 patients (39%).

All patients had a sustained engraftment as defined by neutrophil counts more than 0.5 × 109/L and untransfused platelet counts more than 20 × 109/L for at least 3 consecutive days. The median time for an absolute neutrophil count of 0.5 × 109/L was 14 days (range, 8-18 days); median time for platelet count more than 20 × 109/L was 11 days (range, 8-17 days). One patient (4%) experienced secondary graft failure at day +150, but she did not receive a second SCT from a different donor because of rapidly progressive disease.

To avoid the binding of alemtuzumab to CD8-depleted DLIs, plasma levels were measured by enzyme-linked immunosorbent assay in the first 10 patients at day 30 after SCT: in only 2 patients was the drug detectable at a lympholytic level (> 0.1 µg/mL).

We assessed chimerism in peripheral blood for all patients at day +30: 26 (92%) had full donor hematopoiesis (≥ 95% donor cells) and 2 (8%) were mixed chimeras. All patients achieved full
after CD8 depletion, were 12.3 x 10^9/kg and 8.3 x 10^9/kg, respectively, which was slightly higher than the median value).

Feasibility of CD8-depleted DLI

Processing with ClinMACS CD8 Microbeads was performed on 28 lymphoprepeses. The median CD4+ T-cell recovery was 84% (range, 31%-130%). CD8-depletion reduced the content of CD8+/CD3+ T cells by a median value of at least 3.3 log (range, 1.7-4). The median CD3+, CD56+/CD16-, and CD19+ cell recovery was 55% (range, 25%-91%), 50% (range, 17%-82%), and 68% (range, 23%-128%), respectively (Table 3). The median number of total CD3+, CD3+/CD4+, CD56+/CD16+, and CD19+ achieved after CD8 depletion was 18.6 x 10^9/kg, 17.4 x 10^9/kg, 3.2 x 10^9/kg, and 2.8 x 10^9/kg, respectively. We also analyzed the total amount of CD4+/CD25+ T cells infused into each patient with CD8-depleted DLI. Recipients experiencing GVHD did not receive a statistically different amount of CD4+/CD25+ T cells (median, 0.26 x 10^9/kg; range, 0.011-1.1 x 10^9/kg vs 0.08 x 10^9/kg, range, 0.001-1.21 x 10^9/kg; P = .4). These CD4+/CD25+ T cells were confirmed FoxP3-positive.

Twenty-three of 28 patients (82%) received CD8-depleted DLI. The total number of infusions was 54, with a median of 2 per patient (range, 1-3). The median dose per patient was 6 x 10^9/kg (range, 2.15 x 10^9/kg). Five patients did not receive the scheduled DLI because of acute GVHD (n = 2), early death for progressive disease (n = 2), and rapid immune reconstitution with T cells more than 1000/mL (n = 1). Interestingly, no systemic acute toxicity or loss of engraftment was observed after CD8-depleted DLI.

No GVHD was observed in the first group of patients (dose level 1) receiving 3 doses of 1 x 10^9/kg CD8-depleted T cells on days +45, +75, and +105. Because of the absence of major complications, dose escalation was started. Only 5 of 11 patients (45%) of the second group (dose level 2) completed the planned 3 doses of 5 x 10^9/kg CD8-depleted T cells because of GVHD (n = 4) or disease progression (n = 2). A patient developed GVHD after all the 3 planned doses. Because this dose level was found to be associated with an unacceptably high incidence of GVHD, the remaining patients (n = 8) received a desescalated schedule (1000 kg day +45, followed by 5 x 10^9/kg on days +75 and +105; dose level 3): 6 patients completed all the infusions without any GVHD; one experienced skin grade 2 acute GVHD; one died of pneumonia and did not receive the day +105 infusion.

Overall, 6 of 25 patients (26%) developed acute GVHD (n = 4 grade II, n = 2 grade III/IV) after DLI, requiring systemic immunosuppressive treatment. Acute GVHD resolved after a brief course of therapy in 2 of them. Chronic GVHD was observed in 4 patients (limited, n = 1; extensive, n = 3) and was de novo in only one patient; the limited form resolved after topical steroids.

Evaluation of T- and B-cell immune reconstitution

We observed a statistically significant increase of circulating CD3+/CD4+ T cells (median, 107/μL; range, 1-600/μL) at day +120 post-DLI, compared with day +30 (P = .002, unpaired t test) with a higher proportion of CD4+/CD45RO+/CD45RA- versus CD4+/CD45RO-/CD45RA+ T cells (88% vs 12%). At day +120, the expansion of CD3+/CD8+ was not significant (compared with the day +30 value) with a median value of 23/μL (range, 2-490/μL), which expressed the CD11a/CD54 memory/effector phenotype (P = .07). NK-cell levels did not change significantly before and after DLI. CD19+ B cells increased from a median of 10 cells/μL (range, 2-100 cells/μL) at day +30 to 135 cells/μL at day +120 (range, 6-300 cells/μL) (P = .01; Figure

Table 2. Main infections after engraftment

<table>
<thead>
<tr>
<th>Infections</th>
<th>No. (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral infections</td>
<td></td>
</tr>
<tr>
<td>CMV replication</td>
<td>25 (42)</td>
</tr>
<tr>
<td>CMV disease</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hepatitis zoster</td>
<td>5 (18)</td>
</tr>
<tr>
<td>EBV reactivation</td>
<td>5 (18)</td>
</tr>
<tr>
<td>PTLD</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Fungal infections</td>
<td></td>
</tr>
<tr>
<td>Lung-aspergilosis</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Pneumonia/Condidi</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Bacterial infections</td>
<td></td>
</tr>
<tr>
<td>Gram-positive</td>
<td>3 (22)</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>12 (43)</td>
</tr>
</tbody>
</table>

CMV indicates cytomegalovirus; EBV, Epstein-Barr virus; and PTLD, posttransplant lymphoproliferative disorder. Some figures and tables have been transcribed accurately, with all relevant information presented.
Table 3. CD8 depletion: values before and after processing of mononuclear cells and lymphocyte subsets

<table>
<thead>
<tr>
<th></th>
<th>MNC ( \times 10^9/\mu L )</th>
<th>CD8(^+) ( \times 10^9/\mu L )</th>
<th>CD4(^+)/CD8(^+) ( \times 10^9/\mu L )</th>
<th>CD4(^+)/CD19(^+) ( \times 10^9/\mu L )</th>
<th>CD19(^+) ( \times 10^9/\mu L )</th>
<th>CD8(^+)/CD16(^+) ( \times 10^9/\mu L )</th>
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</thead>
<tbody>
<tr>
<td>Median before</td>
<td>90.6 (25-141)</td>
<td>51.4 (16-116)</td>
<td>22.5 (11-42)</td>
<td>11 (6-31)</td>
<td>4.5 (2.5-11)</td>
<td>5.8 (2.3-23)</td>
</tr>
<tr>
<td>Median after</td>
<td>25.5 (11-111)</td>
<td>16.8 (5-41)</td>
<td>17.4 (5-69)</td>
<td>2.0 (0.001-2.200)</td>
<td>2.8 (1.4-12.5)</td>
<td>3.2 (0.5-12)</td>
</tr>
<tr>
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<td>( P )</td>
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<td>&lt; .001</td>
<td>.01</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

MNC indicates mononuclear cells.

2). The median numbers of CD3\(^+\)/CD4\(^+\), CD3\(^+\)/CD8\(^+\), CD19\(^+\), and CD56\(^+\) cells were 187/\( \mu L \), 120/\( \mu L \), 186/\( \mu L \), and 813/\( \mu L \) at day 150, respectively.

We performed a comparative analysis between the dose level 1 and 3 groups: the median value of circulating CD4\(^+\) T cells at day +120 was 57/\( \mu L \) versus 136/\( \mu L \), respectively \( (P = .03) \). No statistically significant difference was observed in the subsequent time points. We observed a trend for a better immune reconstitution at dose level 3 also for CD19\(^+\) cells: the median value of circulating CD19\(^+\) at day +120 was 37/\( \mu L \) (dose level 1) versus 105/\( \mu L \) (dose level 3: \( P = .08 \)). Of note, circulating T cells in the dose level 2 group were very low because of the immunosuppressive therapy with corticosteroids (5 patients had acute GVHD, thus preventing any comparison with the other dose levels).

In 20 patients (72%), TREC counts per microgram (TRECs/\( \mu g \)) of DNA were determined by quantitative PCR in PB lymphocytes, with a median observation time of 360 days (range, 180-990 days). The median value of TREC/\( \mu g \) of DNA was 2.2 \( \times 10^8 \) (range, 0.4-14 \( \times 10^8 \)) in 15 normal donors with the same median age. TREC counts were very low before SCT in the majority of patients (75%). Median baseline TREC values were lower also in patients younger than 50 years (median value, 0.4 \( \times 10^7 \)). Ten of 20 (50%) patients showed measurable TREC at 1 year with a median value of 1.3 \( \times 10^7 \) TREC/\( \mu g \) of DNA (range, 0.6-18 \( \times 10^7 \); Figure 3). Nine of 10 patients with measurable TREC were younger than 50 years and are alive.

In 10 patients, TCR spectratyping was performed before and at days +180 and +360 after SCT. The TCR complexity score was evaluated as previously reported by Bomberger et al.\(^{14} \); in a control group of 10 normal donors, the median TCR complexity was 98% (range, 85%-109%). Before conditioning, most patients had a Gaussian-like TCR-\( \beta \) repertoire pattern (median, 97% TCR complexity; range, 82%-112%). At 1 year after SCT, median TCR complexity was 70% (range, 49%-120%); Figure 3). All the patients showing posttransplantation thymopoiesis had a high TCR complexity score at 1 year after SCT.

IgH CDR3 spectratyping has been analyzed in 9 patients: a partial return to a polyclonal repertoire at 1 year after SCT was observed. Median number peaks at day 360 in Vh1, Vh2, Vh3, Vh4a, Vh4b, Vh5, and Vh6 were 21, 16.5, 17, 15, 15.5, 17, 16.5, and 20 in normal donors and 11.5, 10.5, 12.5, 10.5, 8.5, 13, 7.5, and 16.5 in patients (Figures 4 and S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). The majority of the patients achieved normal level of IgM at 6 months after SCT (median, 94 mg/dL; range, 27-230 mg/dL); only at 1 year after SCT, the majority of the patients had normal values of IgG and IgA: median, 746 mg/dL (range, 440-1500 mg/dL), median 78 mg/dL (range, 50-161 mg/dL).

CMV- and EBV-specific T cells after CD8-depleted lymphocyte infusions

After SCT, we examined functional immune recovery in 6 patients of dose level 3, longitudinally from day +45 until day +450 (Figures 5, S2). To confirm the specificity of antigen-induced CD137 expression, we first analyzed PBMCs from CMV-negative (\( n = 5 \)) and CMV-positive healthy donors (\( n = 5 \)) stimulated with CMV peptides: the median frequencies of CMV peptide-reactive CD137\(^+\)/CD8\(^+\) and CD137\(^+\)/CD4\(^+\) T cells were 0.2% (range, 0.1%-0.4%) and 0.3% (range, 0.1%-0.6%), and 2.5% (range, 0.1%-0.4%).
2.1%-3.5%) and 1.4% (range, 1%-2%), respectively. The median frequencies of CD137+/CD8+ and CD137/CD4+ T cells in donors after activation with CytoStim (Miltenyi Biotec), were 10.2% (range, 4%-15.2%) and 14% (range, 9%-15.8%), respectively. Responses to CytoStim (Miltenyi Biotec) were detected for both CD8+ and CD4+ T cells in all the patients analyzed (median CD137+/CD8+ and CD137+/CD4+ T cells were 4% [range, 1%-7%] and 4.5% [range, 1%-8.5%], respectively).

Before CD8-depleted DLI, we did not observe any CMV response in all 6 patients analyzed. Only 2 of 3 CMV+ patients were allografted from CMV+ donors, and CD8 T-cell responses were observed after CD8-depleted DLI; the frequencies were 2.4% and 2.0%, respectively, at day +200 after SCT. In 2 other patients (R CMV+/D CMV-), the frequency of CMV-specific CD8+ T cells was 3.7% and 2% at day +200. All the aforementioned patients did not experience further episodes of CMV reactivation. In the last patients analyzed (R CMV+/D CMV-), the frequencies of CMV-specific CD8+ T cells were less than 0.5%. The expansion of CMV-specific CD8+ T cells in 4 of 6 patients exceeded the one of CD4+ T cells (median frequencies CD4+/CD8+; 0.9% range, 0.1%-1.4%), although frequencies of CD4+/CD137+ T cells tended to increase progressively starting from day +200.

EBV-specific T cells (CD8+ and CD4+) against BZLF1 and EBNA1 antigens were evaluated in the same 6 patients from day +100 to day +450. All patients received allografts from EBV+ donors. EBV-specific responses were detected in 4 of 6 of them: the median frequencies of BZLF1- and EBNA1-specific CD3+/CD8+ T cells were 2.1% (range, 0.5%-5%) and 1.5% (range, 0.3%-2.5%), respectively. Only 2 patients showed an early response to BZLF1 at day +200; 1 patient showed a concomitant expansion of CD4+ and CD8+, the other one showed an expansion of CD4+ only (Figure S3).

OS, progression-free survival, and disease response to CD8-depleted lymphocytes

At a median follow-up of 28 months (range, 6-65 months), 12 patients (45%) were alive and 16 died of disease (n = 10); NRM (n = 6). Nine of 12 patients were in complete remission (n = 1 chronic lymphocytic leukemia (CLL), n = 2 HL, n = 3 aggressive lymphomas of T phenotype, n = 1 follicular cell lymphoma, n = 1 multiple myeloma, and n = 1 acute myeloid leukemia; Table 4). The estimated 2-year OS and progression-free survival were 44% (95% confidence interval, 26%-62%) and 45% (95% confidence interval, 25%-63%), respectively (Figure 6). Patients with chemoresistant disease had a better outcome: 2-year OS 75% versus 30% of the chemoresistant group (P < .004).

Disease relapse or progression occurred in 14 of 28 (30%) patients at median time of 4 months after SCT (range, 1-11 months), causing death in 10 of them (n = 4 aggressive NHL, n = 1 CLL/Richter, n = 4 HL, n = 1 multiple myeloma). Four patients with refractory disease (n = 1 acute myeloid leukemia, n = 2 HL, n = 1 CLL) responded to CD8-depleted DLI; all these patients achieved complete remission coincident to a GVHD flare (median duration of response, 14 months; range, 7-19 months), but unfortunately they died because of disease progression (n = 1) or toxicity (n = 3).

Discussion

In this prospective phase 1/2 trial, we showed that (1) RIC regimen allows a stable engraftment of haploidentical stem cells with a
limited incidence of acute GVHD; (2) younger patients with chemoresistant lymphoid malignancies can attain a long-term disease control; and (3) CD8-depleted DLIs are feasible and promote immune reconstitution.

Myeloablative haploidentical SCT with megadoses of CD34+ cells and no GVHD prophylaxis, pioneered by the Perugia group, had some limitations, including graft failures, an NRM rate ranging from 25% to 40%, and the occurrence of organ toxicity related to high-dose conditioning in poor-risk patients. For these reasons, the use of RIC regimens and the new methods for graft manipulation became an appealing area of investigation. Rizzieri et al. used a RIC regimen, unmanipulated grafts, and GVHD prophylaxis, reporting a 63% 1-year OS in standard-risk patients. More recently, a strategy including a RIC regimen followed by CD3/CD19-depleted grafts has been proposed as an alternative option for haploidentical transplantations. Despite the rapid and sustained engraftment and the accelerated immune reconstitution, a relevant incidence of acute GVHD was reported.

Although the escalation of the stem cell dose played a crucial role to favor a high engraftment rate, graft failures ranging from 5% to 14% have been frequently reported after haploidentical SCT. In our study, only one secondary graft failure was observed; probably the pretransplantation screening for anti-HLA antibodies has limited this complication. Indeed, the detection of donorspecific HLA class I and II antibodies was associated with engraftment failure in unrelated transplantsations. Despite the inclusion of poor-risk advanced malignancies, an encouraging 26% NRM rate and 44% OS at 2 years were observed in our study. Interestingly, late NRM was restricted only to 2 patients dying 1 year after SCT. Possible explanations for the promising clinical results are (1) RIC could have reduced acute organ toxicity and thymic damage; (2) drugs used in the conditioning regimen as well as alemtuzumab had a significant antitumor activity; and (3) low-dose alemtuzumab allowed the use of early DLI add-backs.

The occurrence of defective immune recovery after haploidentical SCT was associated with a high risk of severe infections, which heavily affected morbidity and mortality. Nonetheless, adoptive immunotherapy was investigated mostly in children who have a functional thymus and lower incidence of GVHD compared with adults. Azaroliz et al. demonstrated that the infusion of low-dose donor T cells, depleted of alloreactive lymphocytes, significantly increased CD4+ and CD8+ T cells, accelerating CMV- and EBV-specific immunity in the first 4 months after SCT. In adults, data concerning the use of T-cell add-backs are very limited. Perruccio et al. described the immune recovery in myeloablative haploidentical SCT, indicating that (1) CD4+ cell counts were inferior to

Table 4. Patient outcomes

<table>
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<tr>
<th>UPN</th>
<th>Status at SCT</th>
<th>Day of relapse</th>
<th>OS</th>
<th>Day*</th>
<th>Cause of death</th>
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SCT indicates stem cell transplantation; PD, progressive disease; MR, minimal response; RR, complete remission; PR, partial remission; NHL, non-Hodgkin lymphoma; GLI, chronic lymphocytic leukemia; PTLD, posttransplantation lymphoproliferative disorder; HL, Hodgkin lymphoma; MM, multiple myeloma; GVHD, graft-versus-host disease; and —, not applicable.

*Day of last follow-up.

Figure 5. Percentage value of CD4+/CD137+ and CD8+/CD137+ after stimulation of patients' PBMCs with a CMVpep99 peptide pool at different time points after haploidentical stem cell transplantation. Positive and negative control ranges are given in the text.

Figure 6. Kaplan-Meier plot of estimated progression-free survival and overall survival for all the patients.
In the clinical setting, haploidentical SCT has been usually employed for salvage of acute myeloid leukemias, and few data on its use in lymphomas are available. Sykes et al\(^{20}\) reported a very small study in which 2 of 5 lymphoma patients with refractory disease had a clinical response. In addition, Burroughs et al\(^{21}\) in patients with Hodgkin lymphoma, obtained an encouraging 69% progression-free survival. Our study reports the data on a larger number of lymphoma patients and shows that those with chemosensitive disease had a better outcome and could experience long-term remissions. Larger prospective trials are required before considering this promising therapeutic option as a routine salvage strategy for advanced hematologic malignancies.

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Authorship

Contribution: A.D. analyzed and interpreted the data and wrote the paper; C.C. performed experiments, analyzed data, and revised the paper; A.R., A.V., and S.D.T. performed experiments; L.F., E.S., and C.C.-S. included data of patients treated; M.M. and P.L. performed cell separation and immunophenotype analysis; L.G. performed radiotherapy; C.L. performed HLA analysis; and P.C. conceived the study, interpreted the data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Cellular Immune Reconstitution and Its Impact on Clinical Outcome in Children with β Thalassemia Major Undergoing a Matched Related Myeloablative Allogeneic Bone Marrow Transplant

Reena Rajasekar, Vikram Mathews, Kavittha M. Lakshmi, Biju George, Auro Viswabandya, Mammen Chandy, Alok Srivastava

We have prospectively analyzed cellular immune reconstitution (IR) in 63 consecutive pediatric patients with β thalassemia major who underwent an HLA matched related allogeneic bone marrow transplant (BMT). Samples from bone marrow graft and posttransplant peripheral blood samples from recipients at specified time points were assessed for IR of cellular subsets. The median age of the cohort was 7 years, and there were 37 (59%) males. A CD34 cell dose above the median value of 7.3 × 10⁶/kg had a lower incidence of bacterial (P = .003) and fungal (P = .003) infections in the posttransplant period, and was not associated with an increased risk of graft-versus-host disease (GVHD). Among cases that did develop grade II-IV GVHD the absolute CD8 (116 versus 52 cells/µL, P = .012), CD8 naïve (74 versus 9 cells/µL, P = .005), and CD8 memory counts (44 versus 21 cells/µL, P = .010) were significantly higher on day 15. Fifteen patients (24%) rejected their graft (7 primary and 8 secondary). The day 28 natural killer (NK) cell count was significantly associated with secondary graft rejection, event-free survival (EFS), and overall survival (OS) (P = .044, .013, and .034, respectively). On a multivariate analysis, patients with a day 28 NK cell count below the median value of 142/ µL had a significantly higher rejection rate (hazard ratio [HR] = 11.1, P = .038) and a lower EFS (HR = 16.3, P = .034).

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KEY WORDS: Immune reconstitution, Bone marrow transplantation, Thalassemia major, NK cell reconstitution, Graft rejection

INTRODUCTION

An allogeneic stem cell transplant (SCT) remains the only curative option for numerous malignant and inherited hematologic conditions. The clinical efficacy of an SCT is limited by regimen-related toxicity secondary to the conditioning regimen, immune response by donor cells to recipient antigens resulting in graft versus host disease (GVHD) and delayed or inadequate immune reconstitution leading to infections, recurrence of a malignancy, and occasionally rejection of the graft [1,2].

Although immune reconstitution post-SCT has been extensively studied in adults, there is limited data in the pediatric population [1]. An early low plasmacytoid dendritic cell count has been reported by us and others to be associated with acute and chronic GVHD (aGVHD, cGVHD) [3,4]. Rapid lymphocyte and lymphocyte subset recovery have been reported to be associated with a favorable outcome [5-7]. It is known that the ability to generate CD45RA⁺ (naive T helper cells) decreases with age, because this is thymic dependent [8,9]. It is also recognized that there is earlier generation of helper T cells and B cells in children than in adults [1]. There is, however, limited data on the impact of these variations in the pattern of immune reconstitution on clinical outcomes post-SCT in a uniform cohort of children.

Natural killer (NK) cells are innate immune cells critical to host defense against invading pathogens and malignant transformation. NK cells have been extensively studied in the posttransplant period, as they are potentially associated with both rejection and a graft-versus-leukemia (GVL) effect [10,11]. Experimental data suggests that they have a number of
potentially beneficial effects, including an NK cell-versus-leukemia effect reducing relapse, NK cell-versus-residual host T cell reducing graft rejection rate, and NK cell-versus-host dendritic cells, potentially reducing the risk of GVHD [10,12,13]. In the pediatric population with high-risk thalassemia major, graft rejection is a major problem, and can occur in 20% to 50% of cases [14]. The impact of the pattern of NK cell reconstitution in this population has not been studied.

We prospectively analyzed the effect of immune reconstitution in a pediatric population of patients with β thalassemia major undergoing a myeloablative HLA matched related allogeneic SCT.

PATIENTS, MATERIALS, AND METHODS

This prospective study included all consecutive patients who underwent an allogeneic HLA matched related SCT for β thalassemia major at our center, between December 2003 and December 2006. All patients had 6 antigen HLA-matched sibling or family donors. A written informed consent was obtained from parents or legal guardians for all patients.

Pretransplant Evaluation

All patients were evaluated with a complete blood count (CBC), biochemical profile, and serology for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), and cytomegalovirus (CMV). A liver biopsy was performed at the time of Hickman catheter insertion.

Conditioning

Patients were conditioned using a conventional myeloablative regimen consisting of busulfan (Bu) from 16 mg/kg (1 mg/kg/dose 4 times daily × 4 days) administered orally (dose adjustment based on pharmacokinetic levels was not attempted), cyclophosphamide (Cy) 200 mg/kg given over 4 days (50 mg/kg/day i.v. over 1 hour), and antilymphocyte globulin (Pasteur Merieux, Lyon, France) or antithymocyte globulin (ATG; Pharmacia and Upjohn, Kalamazoo, MI) 30 mg/kg/day for 3 days as previously reported [15]. In 25 patients, a new preparative regimen of fludarabine (Flu; 150 mg/m² over 5 days), Bu (14-16 mg/kg over 4 days), and Cy (160 mg/kg over 4 days) was used.

Graft Source and Engraftment

The stem cell source was bone marrow (BM) for all except 2 cases. BM was harvested under general anesthesia from the iliac crest and the target cell dose was 3 x 10^6 nucleated cells/kg body weight of the recipient. Unprimed BM was used in majority of the patients (59%). Twenty-four (38%) patients received granulo-
cyte colony stimulating factor (G-CSF) primed BM (G-CSF 10 µg/kg/day × 2 days prior to BM harvest).

GVHD Prophylaxis

Cyclosporine (CsA) and a short course of methotrexate (MTX) was used as GVHD prophylaxis; CsA was administered at a dose of 2.5 mg/kg i.v. over 4 hours twice daily starting on day -4 and changed to oral administration at 5 mg/kg twice daily when mucositis had resolved. CsA levels were monitored and the dose adjusted to achieve a target level of 100-400 ng/mL. CsA was administered for 6 months, and over the next 6 months the dose was gradually tapered and stopped. If there was active GVHD, tapering and continuation of GVHD CsA beyond this period was left to the treating physician’s discretion. The MTX dose was 10 mg/m² on day -1 and 7 mg/m² on days 3, 6, and 11. If mucositis was severe (grade IV) or bilirubin >20 mg/L, the day 6 and day 11 doses were omitted.

Supportive Care

All patients were nursed in a positive pressure HEPA-filtered transplant unit. Prophylactic acyclovir was administered for the first 100 days; it was continued beyond day 100 if patient had GVHD and required additional immunosuppression. Quantitative CMV polymerase chain reaction (PCR) analysis was done from day 30 onward, once in 2 weeks up to day 100, more frequently if there was evidence of CMV reactivation. Trimethoprim-sulphamethoxazole and oral penicillin prophylaxis was initiated after stable engraftment and continued for a year.

Flow Cytometric Analysis

Harvest samples from donors and peripheral blood samples from posttransplant recipients were assessed for stem cells, lymphocyte subsets, and dendritic cells subsets. Posttransplant peripheral blood samples were obtained from patients on day 15, day 28, day 45, 2 months, 3 months, 6 months, 9 months, and 12 months for flow cytometry analysis. Briefly, cells were labeled using a panel of monoclonal antibodies (mAbs) to CD34, CD133, CD3, CD4, CD45RA, CD45RO, CD8, CD25, CD69, CD19, CD56, CD16, CD11c, CD123, HLA-DR, and lineage cocktail antibodies directly conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP) (Becton Dickinson, San Jose, CA), followed by red cell lysis with ammonium chloride. The cells were then washed and analyzed using FACSCalibur (Becton Dickinson, Mansfield, MA). Data analysis was performed using CellQuestPro software (Becton Dickenson). Dead cells were gated out before the final analysis using forward versus side-scatter dot-plots. Lymphocyte subset
percentages were calculated from the lymphocyte gate as appropriate. The absolute lymphocyte subset counts were then calculated from a routine automated leukocyte count (Beckman Coulter LH 750, Fullerton, CA). A previously described method for identification and enumeration of dendritic cell subsets was used [16]. For stem cell enumeration (CD34+) in the graft, ISHAGE gating strategy was performed as described previously [17].

Definitions and Definition of Outcomes

Incidence and severity of GVHD was defined as per established criteria [18]. Time to engraftment for neutrophils was defined as the first of 3 consecutive days on which absolute neutrophil count (ANC) was >500/mm³. Time to engraftment for platelets was defined as the first of 3 consecutive days on which the unsupported platelet count was >20,000/mm³. Primary graft failure was defined by lack of neutrophil engraftment (ANC <0.5 x 10⁹/L) measured for 3 consecutive days by 28 days posttransplant. Secondary graft rejection was defined when initial engraftment was followed by subsequent development of ANC <0.5 x 10⁹/L for 3 consecutive measurements, recurrence of transfusion dependence, or paucity engraftment with a hypocellular marrow [19]. Secondary graft rejection was further defined as early (graft rejection within 60 days after initial engraftment) or late (graft rejection occurring after 60 days from SCT and initial engraftment).

The data from pretransplant counts were used as reference range for each of cellular subsets analyzed. Achievement of the median pretransplant cellular subset value was considered an event on a Kaplan-Meier analysis. Patients who died before engraftment or had a graft rejection after transplantation were censored at that time point for immune reconstitution studies.

Conventional Lucarelli risk stratification for patients with β thalassemia major undergoing an allogeneic SCT was used [20]; additionally, a high-risk subset of class III cases as previously described was also utilized for analysis [21]. Event-free survival (EFS) was defined from the time of transplant to an event; an event was primary graft failure, death, or recurrence of transfusion dependence. Stable mixed chimerism with transfusion independence was not considered an event for this analysis. Overall survival (OS) was defined as the time from transplant to death from any cause.

Bacterial infection was defined as positive culture from blood and any other sites (urine, sputum, pus, abscess, and catheter). Bacterial pneumonia was also diagnosed when there were clinical and radiologic signs of pneumonia with or without positive sputum culture or a pneumonia that improved after antibacterial therapy [22]. CMV infection was defined as 2 consecutive positive PCR assays within 1 week or a CMV copy number >140 copies/mL whole blood performed using artus CMV PCR Kits (Qiagen, Hilden, Germany). CMV disease was defined as the demonstration of CMV by biopsy specimen from visceral sites (by culture or histology) or the detection of CMV by culture in broncho-alveolar fluid in the presence of new or changing pulmonary infiltrates [23]. All fungal infections were documented as probable, possible, and proved fungal infections based upon the CDC criteria [24].

Statistical Analysis

For comparison of dichotomous variables, a χ² test was done while continuous variables were compared using either a Student's t-test or a Mann-Whitney U-test as was deemed appropriate. The probability of survival was estimated using Kaplan-Meier method for rejection, EFS, and OS, and compared by the log-rank test. Cox models were used to assess the proportional hazards of various subsets both in the graft and those engrafted after transplant on clinical outcomes such as rejection, GVHD, day 100 treatment-related mortality (TRM), EFS, and OS. To confirm outcomes and to adjust for potential confounding factors, multivariate Cox proportional hazards models were also performed. Kinetics of lymphocyte recovery was also evaluated by calculating area under curve (AUC) using trapezoidal method [25]. For all the tests, a value of P < .05 was considered statistically significant. Statistical analyses were performed using SPSS for Windows 11.01 version (SPSS Inc., Chicago, IL).

RESULTS

Demographic and Baseline Characteristics

A total of 63 patients with β thalassemia major underwent an SCT between December 2003 and December 2006 at our center. The median age of this cohort was 7 years (range: 2-14 years). There were 37 (59%) males and 25 (41%) females. By conventional risk stratification [20], there were 7 (11%), 25 (40%), and 31 (49%) transplants in class I, II, and III, respectively. The baseline patient and graft characteristics are summarized in Table 1. The median pretransplant value of the total count and all the subsets analyzed except for dendritic cell count was significantly lower than age matched donors and that of age-matched children as reported previously (supplementary data) [26,27]. Achievement of the median pretransplant cellular subset value was considered an event on a Kaplan-Meier analysis. Patients who died before engraftment or had a graft rejection after transplantation were censored at that time point for immune reconstitution studies.
Table 1. Baseline Patient and Graft Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>7</td>
<td>(3-14)</td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>(63)</td>
</tr>
<tr>
<td>Donor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>8</td>
<td>(3-39)</td>
</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>(43)</td>
</tr>
<tr>
<td>Sex mismatch transplant</td>
<td>40</td>
<td>(64)</td>
</tr>
<tr>
<td>Lucarell class III</td>
<td>31</td>
<td>(49)</td>
</tr>
<tr>
<td>Spleenectomy</td>
<td>2</td>
<td>(3)</td>
</tr>
<tr>
<td>High risk (age ≥ 7 years and liver size ≥ 3 cm) [11]</td>
<td>6</td>
<td>(10)</td>
</tr>
<tr>
<td>TNC (x10⁹/L)</td>
<td>4.4</td>
<td>(1.7-22.7)</td>
</tr>
<tr>
<td>Conditioning regimen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BuCy /ATG</td>
<td>33</td>
<td>(53)</td>
</tr>
<tr>
<td>BuCy /ALG</td>
<td>4</td>
<td>(6)</td>
</tr>
<tr>
<td>BuCy</td>
<td>1</td>
<td>(1)</td>
</tr>
<tr>
<td>Flud /BuCy</td>
<td>25</td>
<td>(39)</td>
</tr>
<tr>
<td>GVHD Prophylaxis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSA + MTX</td>
<td>62</td>
<td>(90)</td>
</tr>
<tr>
<td>CSA + Melphalanavudione</td>
<td>1</td>
<td>(2)</td>
</tr>
<tr>
<td>Graft source</td>
<td>BM = 37; GBM = 24; PBSC = 2</td>
<td></td>
</tr>
</tbody>
</table>

Table indicates Bu = Busulfan; ATG = antithymocyte globulin (Pharmacia Upjohn); ALG = antilymphocyte globulin (Pasteur-Merieux); Cy = Cyclophosphamide; Flud = Fludarabine; CSA = Cycosporine; MTX = Methotrexate; BM = bone marrow; PBSC = peripheral blood stem cells.

The median BM cell dose was 4.4 x 10⁹ (range: 1.7-22.7) total nucleated cells/kg and 7.3 x 10⁶ CD34/kg (range: 1.2-23). The total WBC count in the harvested product was higher in G-CSF primed BM (BM = 19.7 x 10⁹/L vs. G-BM = 37.75 x 10⁹/L, P = .000), resulting in a lower volume of marrow harvested to achieve the target cell dose of 3 x 10⁹ total nucleated cells/kg (BM = 430 mL versus G-BM = 280 mL, P = .000). The graft characteristics were similar in both BM and G-CSF-primed BM sources for stem cell content (CD34, CD133), but significantly differed in NK, CD19, CD8CD45RA, and plasmacytoid dendritic cell content (Table 2).

Clinical Outcomes

Of the 63 transplants, 2 patients had peritransplant deaths related to regimen-related toxicity (RRT). Among the 61 patients who could be evaluated for engraftment, 54 (89%) achieved sustained engraftment. Seven patients (11%) had primary graft failure. Five of these patients died prior to day 28 (time point for the first chimerism analysis). Two patients had autologous reconstitution by day 20, and chimerism analysis on day 28 revealed a complete absence of a donor band; both are transfusion dependent and have remained alive on follow-up for more than a year. Of the 54 who had achieved sustained engraftment, 2 (4%) had early (>28 days and <60 days posttransplant) and 6 (11%) had late secondary graft rejections (>60 days posttransplant). On day 28, the time point when the first chimerism analysis was done, 7 of these patients had a mixed chimerism pattern with a median donor band of 79% (range: 26-91), whereas 1 patient had documented complete chimerism (100% donor band) at this time point. The median time to graft rejection among these patients was 77 days (range: 53-184 days).

Among the 54 who had engrafted, 2 died, 1 each on day 17 and day 23 secondary to RRT complicated by infections (prior to documentation of chimerism status). Of the remaining 52 patients who could be evaluated for aGVHD, 8 (15%) developed aGVHD grade (II-IV), whereas 5 of 45 (11%) patients surviving >100 days developed cGVHD. The Kaplan-Meier 3-year EFS and OS for the entire cohort was 67% ± 6% and 78% ± 5%, respectively, at a mean follow-up of 37 months. The EFS and OS according to risk stratification was, class I: 86% ± 13% and 86% ± 13%; class II: 75% ± 9% and 83% ± 8%; class III: 57% ± 9% and 66% ± 10%, respectively.

Immune Reconstitution Post-SCT

The data from pretransplant counts were used as reference ranges for each of the cellular subsets analyzed. Achievement of the median pretransplant cellular subset value was considered an event on a Kaplan-Meier analysis. NK cells were the first cells to recover, within a month, in the early posttransplant period. Following immune reconstitution of NK cells, there was a transient increase in NK cell numbers above pretransplant baseline levels, and thereafter remained within the normal range throughout the rest of the posttransplant period (Figure 1). Dendritic cells (monocytoid and plasmacytoid) also recovered by day 30 posttransplant and thereafter remained within the normal range. The Kaplan-Meier estimate of the mean time for CD3 recovery is 9.7 months (95% confidence interval [CI]: 8.5-12). Recovery of CD4 cells to baseline levels is even further delayed with the mean Kaplan-Meier estimate not being achieved in the 1 year study period of this analysis. At the end of 1 year, IR analysis revealed that only 7 of 54 (13%) patients who had sustained engraftment achieved the pretransplant median CD4 levels. However, CD8 cells reconstitute fairly rapidly with a median Kaplan-Meier estimate of 2 months (95% CI: 0-4 months) resulting in an inverted CD4/CD8 ratio for a prolonged period following transplantation. CD4⁺CD45RA⁺ (helper naive T cells) subset recovery is delayed more than a year, whereas CD8⁺CD45RA⁺ (cytotoxic naive T cells) normalize within the first month post-SCT. There was a correlation with the donor age and speed of recovery of CD4⁺CD45RA⁺ T cells, with younger age of the donor being significantly associated with a faster recovery (r = .3; P = .032). Kaplan-Meier estimates showed that the median time for 6 cell (CD19) reconstitution was 4 months (95% CI: 1-7 months).

There were too few and insignificant variations in immunosuppression withdrawal to comment on the
impact this could have had on immune reconstitution. The immune reconstitution data of this cohort is summarized in Figures 1 and 2.

Factors Influencing Immune Reconstitution Post-SCT

Stem cell source and conditioning regimen

The Kaplan-Meier estimates of NK, cytokine induced killer cells (CIK-CD3-CD56+CD16+), B and T cell subsets (CD4, CD4D45RA, CD4D45RO, CD4DCD25, CD8, CD8CD45RA, CD8CD45RO, and CD8CD25), and plasmacytoid dendritic cells recovery were not significantly different between transplants utilizing either a BM or G-BM graft source. However, the AUC values of NK, B, T cell subsets (except for CD8, CD8CD45RO, and CD8CD25), and dendritic cells were all significantly higher in patients receiving G-BM compared with BM grafts, although this did not translate into a significant improvement in EFS, OS, or reduction in the incidence of infections (Table 2). These graft sources also differed in reconstitution of monocyctic dendritic cells. The day 30 Kaplan-Meier estimate for MC recovery was 32% ± 10% and 18% ± 9% for BM and G-BM, respectively (P = 0.029). There were no significant differences in the immune reconstitution patterns among patients who received antilymphocyte globulin (ALG) as part of the conditioning regimen versus those who did not (Table 3).

Acute GVHD

Patients with aGVHD grade II-IV had higher CD3 (P = 0.012), CD8 (P = 0.002), CD8CD45RA (P = 0.027), and CD8CD45RO (P = 0.005) AUC values compared with those who did not develop GVHD. Among cases that did develop grade II-IV GVHD the absolute counts were significantly higher on day 15 in the following cellular subsets; CD8: 116 versus 52 cells/μL, P = 0.012, CD8CD45RA: 74 versus 19 cells/μL, P = 0.005, and CD8CD45RO: 44 versus 21 cells/μL, P = 0.010. Only 1 of 8 patients who eventually developed aGVHD had evidence of aGVHD at this timepoint.

Infections

Patients who had evidence of viral infections post transplant (within study time period of 1 year) had
a significantly higher CD8CD45RA AUC value ($P = .021$) compared with those that did not. None of the other cellular subsets AUC or absolute values at any time point significantly differed in those who had and those who did not develop a viral infection. Similarly, there was no obvious correlation with cellular subset recovery kinetics and bacterial, fungal, or viral infections.

**Impact of Graft Characteristics and Day 28 Lymphocyte Subset Counts on Clinical Outcomes**

After extensive immune reconstitution analysis and its impact on clinical outcomes, the only time point where differences in cellular subset levels had a significant impact on clinical outcome after engraftment was the day 28 subset values. Eleven of 63 patients were excluded for day 28 subset analysis. Nine died before day 28, whereas in 2 patients a blood sample was not available at this particular time point. Fifty-two patient’s values were available for evaluation at this time point. The day 28 values represent an early period of measurable donor immune reconstitution and could also potentially be a clinically useful early predictor for outcomes after SCT. Hence, further detailed analysis of this time point along with graft characteristics on clinical outcomes was undertaken in this study.
**Engraftment and rejection**

The median time to neutrophil and platelet engraftment was 16 days (range: 10-23) and 26 days (range: 10-49), respectively. Patients with day 28 NK above the median value (>142/μL) had faster platelet engraftment compared with those below the median value (26 versus 30 days, \( P = .031 \)) (Table 4). None of the other cellular subset had an impact on the time to neutrophil and platelet engraftment. Fifteen of 63 patients (24%) rejected their graft; 7 of 15 were primary graft failures and were excluded from day 28 analysis. Among the 7 cases with primary graft failure the median leukocyte counts on day 28 was \( 0.3 \times 10^9/\text{L} \) (range: 0.2-0.8). At this time point and at an earlier (day 15) time point the leukocyte count was too low to make a meaningful assessment of subset cellular immune reconstitution. Of the remaining 8 patients, 2 (13%) had early and 6 (40%) had late secondary graft rejections post-SCT. After excluding primary graft failures (\( n = 7 \)), the median day 28 NK count in patients who rejected their graft (\( n = 8 \)) was significantly lower compared to those who did not reject (\( n = 42 \)) (91/μL versus 150/μL; \( P = .013 \)) as shown in Figure 3.

None of the other cellular subsets at this time point was significantly associated with graft rejection. There was no evidence that graft rejection was preceded or directly related to any infective process. Posttransplant chimerism at day 28 was complete in 37 of 52 patients (71%) with only donor-specific bands. Among the patients with mixed chimera (15/52 = 29%), the donor band contributed a median of 87% (range: 26-98).

**GVHD**

Eight of 52 (15%) patients who could be evaluated developed aGVHD grade (II-IV). Five of 45 patients (11%) surviving >100 days developed cGVHD. None of the subsets that were found to be a risk factor in univariate analysis retained significance for aGVHD (CD4CD45RO and CD4CD25 cell dose) and cGVHD (CD34 cell dose, day 28 activated NK and day 28 CD8CD25 cells) in a multivariate analysis. There was no association of day 28 dendritic cells with development of GVHD. Similarly, neither the graft source (BM or G-BM) or the use of ALG in the conditioning regimen had an
Table 3. Comparison of Graft Characteristics and Clinical Outcomes among Recipient's Who Received Antilymphocyte Globulin in Their Conditioning Regimen versus Those Who Did Not

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ATG/ALG (n = 37)</th>
<th>Without ATG/ALG (n = 26)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNc × 10^9/kg</td>
<td>4.8 (1.7-10)</td>
<td>3.9 (1.7-22.7)</td>
<td>.276</td>
</tr>
<tr>
<td>HSC</td>
<td>7.1 (1.2-17.7)</td>
<td>7.6 (2.9-33)</td>
<td>.660</td>
</tr>
<tr>
<td>CD34+</td>
<td>3.9 (0.5-10)</td>
<td>3.8 (1-16.6)</td>
<td>.785</td>
</tr>
<tr>
<td>CD19+</td>
<td>5.6 (0.1-49.8)</td>
<td>3.4 (1.1-23.3)</td>
<td>.054</td>
</tr>
<tr>
<td>CD25</td>
<td>21.6 (1.1-18.4)</td>
<td>3.5 (0.3-52.5)</td>
<td>.001</td>
</tr>
<tr>
<td>CD33</td>
<td>41.7 (8.9-120.3)</td>
<td>47.1 (7.4-200)</td>
<td>.125</td>
</tr>
<tr>
<td>CD4+</td>
<td>22.5 (4.6-66.3)</td>
<td>22.8 (1.1-133.7)</td>
<td>.295</td>
</tr>
<tr>
<td>CD8+</td>
<td>21.9 (4.2-91)</td>
<td>21.9 (14.4-104.6)</td>
<td>.204</td>
</tr>
<tr>
<td>CD4+CD45RA+</td>
<td>10.2 (0.9-53.5)</td>
<td>10.3 (2.6-66.3)</td>
<td>.577</td>
</tr>
<tr>
<td>CD4+CD45RA*</td>
<td>12.4 (2.2-50.1)</td>
<td>13.7 (4.9-69.4)</td>
<td>.264</td>
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<tr>
<td>CD8+CD62L*</td>
<td>8.0 (1.6-283)</td>
<td>7.2 (3.3-66.5)</td>
<td>.219</td>
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<tr>
<td>CD11c-HLADR+Lin-</td>
<td>6.7 (0.1-43.9)</td>
<td>7.4 (1.3-63.9)</td>
<td>.577</td>
</tr>
<tr>
<td>CD123-HLADR+Lin-</td>
<td>6.5 (0.1-41)</td>
<td>0.3 (0.1-0.8)</td>
<td>.009</td>
</tr>
<tr>
<td>ANC &gt;500/mm^3</td>
<td>1.3 (0.3-5.1)</td>
<td>0.7 (0.1-2.8)</td>
<td>.003</td>
</tr>
<tr>
<td>Platelet &gt;200,000/mm^3</td>
<td>16 (10-30)</td>
<td>17 (1-23)</td>
<td>.155</td>
</tr>
<tr>
<td>Day 28 subsets (×10^3/mL)</td>
<td>224 (41-1220)</td>
<td>486 (47-1344)</td>
<td>.065</td>
</tr>
<tr>
<td>CD3</td>
<td>8 (1-87)</td>
<td>10 (3-39)</td>
<td>.256</td>
</tr>
<tr>
<td>CD4</td>
<td>116 (9-338)</td>
<td>155 (27-545)</td>
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<tr>
<td>CD8</td>
<td>140 (14-967)</td>
<td>313 (4-1134)</td>
<td>.091</td>
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<td>Natural killer</td>
<td>141 (3-539)</td>
<td>142 (27-1718)</td>
<td>.788</td>
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<tr>
<td>MC</td>
<td>11 (1-30)</td>
<td>6 (1-50)</td>
<td>.514</td>
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<tr>
<td>PC</td>
<td>8 (1-37)</td>
<td>5 (1-25)</td>
<td>.497</td>
</tr>
<tr>
<td>Acute GVHD (grade II-IV)</td>
<td>4 (12)</td>
<td>4 (21)</td>
<td>.443</td>
</tr>
<tr>
<td>Chronic GVHD</td>
<td>4 (13)</td>
<td>1 (8)</td>
<td>1.000</td>
</tr>
<tr>
<td>Survival</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary graft rejection</td>
<td>6 (16)</td>
<td>2 (8)</td>
<td>.448</td>
</tr>
<tr>
<td>Death</td>
<td>6 (16)</td>
<td>8 (31)</td>
<td>.223</td>
</tr>
<tr>
<td>EFS at 3 years</td>
<td>76% ± 3%</td>
<td>60% ± 10%</td>
<td>.145</td>
</tr>
<tr>
<td>OS at 3 years</td>
<td>86% ± 5.2%</td>
<td>63% ± 10%</td>
<td>.066</td>
</tr>
</tbody>
</table>

MC indicates monocyctoid dendritic cells; PC, plasmacyctoid dendritic cells; EFS, event-free survival; OS, overall survival; GVHD, graft-versus-host disease; ANC, absolute neutrophil count; TNC, total nucleated cells; HSC, hematopoietic stem cell; ATG, antilymphocyte globulin; ALG, antilymphocyte globulin.

Influence on development of aGVHD and cGVHD (Tables 2 and 3).

Infections

During the posttransplant study period 22% of patients had documented bacterial, 13% had viral, and 11% had fungal infections (proved, possible, and probable), which were microbiologically or clinically documented. Patients who received a higher than median CD34 cell dose (median = 7.3 × 10^9/kg) had lower incidence of bacterial (P = .003) and fungal infections (P = .005), whereas viral infections were not influenced by stem cell dose. The median time to neutrophil engraftment was not different between these groups (17 versus 16 days, P = .53). The protective effect of increasing CD34 cell dose was retained in a multivariate analysis (hazard ratio [HR] = 0.3, P = .044) after adjusting for factors that had significance in univariate analysis such as patient age, high-risk group (age ≥7 and liver size ≥cm), and NK cell dose. Neither the graft source (BM versus G-BM) or the use of ALG in the conditioning regimen had an effect on post SCT infections.

Survival

The 3-year Kaplan-Meier estimate of EFS and OS was 67% ± 6% and 78% ± 5%, respectively, at a mean follow-up of 37 months. Eleven of 63 patients (18%) died of TRM within day 100. Among the 11, 8 (73%) deaths were because of RRT, whereas 3 (27%) died of primary graft failure and secondary infections. None of the cellular subsets, either in the graft or those that reconstituted by day 28 influenced the day 100 TRM. However, the day 28 NK counts were found to be statistically significantly associated with EFS and OS when analyzed as continuous variables in a univariate analysis (P = .013 and .034, respectively).

There was no significant difference in the OS and EFS between BM and G-BM stem cell source. Similarly, there was no correlation with OS and EFS among those receiving ALG versus those that did not (Tables 2 and 3).
### Table 4. Comparison of Low and High Day 28 NK Cell Count Patient Groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Low (≤142/µL) n = 26</th>
<th>High (&gt;142/µL) n = 26</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>7 (2-14)</td>
<td>8 (2-13)</td>
<td>.904</td>
</tr>
<tr>
<td>Males</td>
<td>15 (60)</td>
<td>16 (60)</td>
<td>1.000</td>
</tr>
<tr>
<td>Sex mismatch transplant</td>
<td>10 (39)</td>
<td>9 (35)</td>
<td>1.000</td>
</tr>
<tr>
<td>Lucarelli class III</td>
<td>14 (54)</td>
<td>11 (42)</td>
<td>.379</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>2 (8)</td>
<td></td>
<td>.490</td>
</tr>
<tr>
<td>High risk</td>
<td>3 (12)</td>
<td>2 (8)</td>
<td>1.000</td>
</tr>
<tr>
<td>TNC (x10^3/kg)</td>
<td>4.2 (1.7-10)</td>
<td>4.6 (1.7-22.7)</td>
<td>.441</td>
</tr>
<tr>
<td><strong>CD34</strong></td>
<td>6.7 (1.2-6.9)</td>
<td>8.5 (1.8-23)</td>
<td>.231</td>
</tr>
<tr>
<td><strong>CD133</strong></td>
<td>3.8 (1.1-10.8)</td>
<td>4.4 (0.5-16.8)</td>
<td>.191</td>
</tr>
<tr>
<td><strong>CD3</strong></td>
<td>43.4 (9.9-122)</td>
<td>41.7 (20.8-202)</td>
<td>.334</td>
</tr>
<tr>
<td><strong>CD19</strong></td>
<td>5.1 (0.1-118.4)</td>
<td>18.4 (0.7-61.6)</td>
<td>.044</td>
</tr>
<tr>
<td><strong>CD4</strong></td>
<td>22.1 (4.6-64.3)</td>
<td>24.7 (8.2-132)</td>
<td>.360</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td>21.5 (4.2-63)</td>
<td>21.1 (9.5-104.6)</td>
<td>.297</td>
</tr>
<tr>
<td><strong>NK</strong></td>
<td>6.2 (0.1-15.2)</td>
<td>6.4 (1.1-23.3)</td>
<td>.076</td>
</tr>
<tr>
<td><strong>MC</strong></td>
<td>0.8 (0.1-1.1)</td>
<td>0.6 (0.1-4.1)</td>
<td>.023</td>
</tr>
<tr>
<td><strong>PC</strong></td>
<td>0.8 (0.5-5.1)</td>
<td>1.3 (0.2-4)</td>
<td>.164</td>
</tr>
<tr>
<td><strong>Engraftment (days)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ANC &gt;500/mm^3</strong></td>
<td>17 (1-23)</td>
<td>16 (1-20)</td>
<td>.358</td>
</tr>
<tr>
<td><strong>Platelet &gt;20,000/mm^3</strong></td>
<td>30 (15-49)</td>
<td>26 (10-42)</td>
<td>.031</td>
</tr>
<tr>
<td><strong>Day 28 sublethals (x10^3/µL)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD3</strong></td>
<td>155.4 (40.5-2110.9)</td>
<td>223.1 (142.1-1718.1)</td>
<td>.002</td>
</tr>
<tr>
<td><strong>CD19</strong></td>
<td>7.5 (0.6-86.6)</td>
<td>12.5 (0.8-42.0)</td>
<td>.089</td>
</tr>
<tr>
<td><strong>CD4</strong></td>
<td>75.2 (8.9-433.4)</td>
<td>200.6 (48.6-544.5)</td>
<td>.000</td>
</tr>
<tr>
<td><strong>CD8</strong></td>
<td>72.5 (3.7-1041)</td>
<td>269.3 (85.5-1134)</td>
<td>.002</td>
</tr>
<tr>
<td><strong>MC</strong></td>
<td>5.6 (0.3-50)</td>
<td>11.4 (1.3-38)</td>
<td>.037</td>
</tr>
<tr>
<td><strong>PC</strong></td>
<td>6.4 (0.1-10.9)</td>
<td>9.1 (0.7-37.2)</td>
<td>.070</td>
</tr>
<tr>
<td><strong>Acute GVHD (grade II-IV)</strong></td>
<td>3 (12)</td>
<td>4 (15)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Chronic GVHD</strong></td>
<td>2 (9)</td>
<td>3 (13)</td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Survival</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary graft rejection</td>
<td>7 (25)</td>
<td>1 (4)</td>
<td>.021</td>
</tr>
<tr>
<td>Death</td>
<td>5 (19)</td>
<td></td>
<td>.651</td>
</tr>
<tr>
<td><strong>EFS at 3 years</strong></td>
<td>64% ± 10%</td>
<td>96% ± 4%</td>
<td>.006</td>
</tr>
<tr>
<td><strong>OS at 3 years</strong></td>
<td>70% ± 14%</td>
<td>100% ± 0%</td>
<td>.017</td>
</tr>
</tbody>
</table>

MC indicates monocytoid dendritic cells; PC, plasmacytoid dendritic cells; EFS, event-free survival; OS, overall survival; GVHD, graft-versus-host disease; ANC, absolute neutrophil count; TNC, total nucleated cells; NK, natural killer.

**Impact of Day 28 NK Counts on Clinical Outcomes**

The day 28 NK counts were found to be statistically significantly associated with secondary graft rejection, EFS, and OS when analyzed as continuous variables in univariate analysis (P = .044, .013, .034, respectively). None of the other lymphocyte subsets that constituted post-SCT at this timepoint had an impact on these clinical outcomes. Hence, a further detailed analysis was done based on the day 28 NK cell count. The median day 28 NK cell count for those who could be evaluated (52 patients) was 142 cells/µL (range: 3-718 cells/µL).

Patients were grouped based on median value for day 28 NK cells (≤142/µL) as low and the rest as high group (>142/µL) to calculate relative risk for transplantation outcomes. The low and high day 28 NK (N = 26 each) groups were comparable with regard to age, sex mismatch transplant, Lucarelli class III, high risk class III, conditioning regimen, GVHD prophylaxis regimen, and graft characteristics (CD34, CD133, CD3, CD4, CD8, and NK cell dose) as shown in Table 4. However, the CD19, monocytoid cells (MC) in the graft and engraftment kinetics of CD3, CD4, CD8, and MC cells were significantly different between the 2 groups (Table 4).

**Rejection**

The factors that influenced graft rejection in univariate analysis was low day 28 NK ≤142/µL (HR = 8.2, 95% CI: 1.0-66.7, P = .049) and splenectomy (HR = 46.5, 95% CI: 2.9-743, P = .007). Because only 2 patients had splenectomy, this variable was excluded for multivariate analysis. On a multivariate analysis adjusting for patient age, sex mismatch transplant, Lucarelli class, high-risk class III (age ≥ and liver size ≥5 cm), conditioning regimen, and graft sources only a low day 28 NK (≤142/µL) retained statistical significance for rejection (HR = 11.1, 95% CI: 1.14-106.81, P = .038) (Table 5).

Cumulative incidence of rejection compared by log rank test revealed that the low NK group had a significant risk for secondary graft rejections (P = .019) (Figure 4). The sensitivity and specificity for low day
28 NK count in predicting secondary rejection is 88% and 60%, respectively.

**GVHD**

There was no association of day 28 NK cells with regard to development of aGVHD and cGVHD ($P = .620$ and .736, respectively).

**Survival**

On a multivariate analysis with standard risk factors such as patient age, sex mismatch transplant, Lucarelli class, high risk class III, aged 7 years and liver size $\geq 5$ cm, conditioning regimen and graft sources, only a low day 28 NK ($\leq 142/\mu$L) retained statistical significance for EFS (HR = 16.3, 95% CI: 1.6-161, $P = .017$) along with patient's age (HR = 0.7, 95% CI: 0.6-1, $P = .034$). Survival curve analysis also showed that a low day 28 NK cell count ($<142/\mu$L) was a risk factor for EFS ($P = 0.006$) and OS ($P = .017$) as illustrated in Figure 4.

**DISCUSSION**

The factors that influenced immune reconstitution in our analysis was similar to those that have been reported previously [28-30]. Our analysis was consistent with previously reported data in which NK cells were noted to recover to normal levels within 1 to 2 months following SCT, and remained the predominant lymphoid subset in the peripheral blood regardless of transplant type, stem cell source and quantity, patient age, and occurrence of GVHD [1]. Similarly, consistent with reports in the literature, CD8+ levels recovered rapidly as their reconstitution is possibly favored by extra thymic origin, whereas CD4+ subset recovery (thymic dependent) is impaired [8]. CD45RO+ T cells are memory cells, which respond in vitro to recall antigens, whereas CD45RA+ T cells are naive cells, recently issued from the thymus. It has been reported that children have faster recovery of CD45RA+ T cells than adults because of the potential for thymic rebound posttransplantation, and this in turn, could contribute to the reduced risk of infections in children in the posttransplant period [2,3,1,32]. In this study recovery of CD4+CD45RA+ naive T cells was slow, consistent with the pace of recovery that has been reported in the literature [1], and remained below normal levels for the period of this study (1 year), whereas CD8+CD45RA+ naive cells normalized within the first month posttransplant. Previous reports had suggested that the younger the donor age the faster the recovery of CD4+ CD45RA+ T cells [31]. In this analysis we have also noted a similar correlation between donor age and CD4+CD45RA+ T cell recovery, with transplants involving younger donors having a faster recovery of this subset (r = 3, P = .032). Although we did notice a decreased risk for bacterial and fungal infections among patients who received a higher stem cell dose, we could not correlate this to faster CD4+CD45RA+ T cell recovery (data not shown).

Both dendritic, subsets, namely, monocytoid and plasmacytoid cells, in our analysis recovered within a month following transplant, which is consistent with a previously reported data [33]. We and others have reported that in the setting of a peripheral blood SCT a low day 28 plasmacytoid dendritic cell count was associated with aGVHD and cGVHD [3,4]. In this study we were unable to demonstrate a similar effect. This could be because of the small numbers that actually developed GVHD, or it could be that low day 28 plasmacytoid DC count is not predictive of GVHD when the graft source is bone marrow. Larger studies will be required to clarify this further.

Data from this analysis suggests that in this cohort of patients, increasing the stem cell dose reduces the risk of posttransplant bacterial and fungal infections. We hypothesize that faster immunologic recovery occurs with higher CD34 cell doses, and consequently, diminishes the risk of bacterial and fungal infections as observed in a previous report [34]. However, we were not able to demonstrate a correlation in speed of recovery of any specific cellular subset in relationship to the stem cell dose. We also noted that patients in the highest quartile of the stem cell dose did not
have an increased risk of aGVHD or cGVHD (data not shown). Although the number of events is few and the cohort studied small, it would still be reasonable in future to target a CD34 cell dose of $10 \times 10^6$/kg or an MNC dose of $6 \times 10^7$/kg in the graft (lower limit of the fourth quartile values of graft cell dose) in these patients. At these doses, our data would suggest that there should be a significant reduction in posttransplant bacterial and fungal infections without an increased risk of GVHD.

Studies in major histocompatibility complex (MHC) mismatched transplants done in mice and humans have shown that donor NK cells target hematopoietic tissues of the host, eliminating host antigen-presenting cells (APCs) and exerting a GVLM effect against leukemia [35]. A similar effect has been noted with the NK cell dose in the allograft, a higher dose of NK cell infusion being associated with a lower risk of GVHD even in matched sibling transplants [36]. Savani et al [13] have demonstrated that rapid NK cell recovery (NK >150/μL around day 30) as an independent determinant predicting less relapse and better survival after T cell-depleted matched SCT in patients with myeloid malignancies. Previously, the same group had shown that the day 30 NK cell count was a surrogate marker for rapid molecular remission in CML patients [37]. Matthes-Martin et al [38] highlighted the role of NK cells during the early posttransplant period. This group has shown a strong correlation of secondary graft rejection and detection of recipient NK cells on day 28. Our observations in this cohort of patients are consistent with some of the above reports.

Among the lymphocyte subsets at day 28, a prominent role for NK cell reconstitution was noted. Our data suggests for the first time that the absolute numbers of NK cells after allogeneic SCT for patients with β thalassemia major is a strong predictor of secondary rejection and EFS. We have noted that patients who had less than the median number of NK cells on day 28 had a higher incidence of rejection and an inferior EFS and OS. It is important to emphasize that our low and high day 28 NK patient groups (n = 26 each) were comparable with regard to recipient age, sex mismatch transplants, lucarelli Class III, and graft characteristics (CD34, CD133, CD3, CD4, CD8, and NK cell dose). However, there were differences between these 2 groups for the CD19 and monocyteid dendritic cell (MC) dose in the graft, and there were significant differences in the engraftment kinetics of CD3, CD4, CD8, and MC cells between the 2 groups; none of these differences except for a low day 28 NK cell count was associated with rejection in a multivariate analysis. Among patients who had a secondary graft rejection we attempted but failed to demonstrate a subset cellular immune reconstitution parameter beyond day 28, which could predict eventual graft rejection. A limitation of this study was that in the patients who did reject their grafts we did not have cellular subset chimerism data. Twenty-five of our patients were treated with a new Flu-based conditioning regimen that could have had an impact on rejection rates. On
multivariate analysis even after adjusting for this variation in conditioning regimen low day 28 NK cell count was significantly associated with secondary graft rejection. On subset analysis a low day 28 NK cell count was significantly associated with reduced EFS and OS in patients who received either of these conditioning regimens (data not shown). Also, neither conditioning regimens were independently associated with secondary graft rejection ($P = .69$).

Identifying potential causes of low peripheral blood day 28 NK counts after SCT among those who reject their graft needs further investigation. We could not find any association between graft characteristics and the day 28 NK cell count nor could we identify any predictors of a low day 28 NK cell count.

Conventional risk stratification for patients with $\beta$ thalassemia major is based on presence of hepatomegaly, evidence of portal fibrosis in the liver, and inadequate iron chelation therapy (Lucarelli class I, II, and III) [20]. This classification has been validated by different groups, and remains the most important prognosticator for patients with $\beta$ thalassemia major undergoing an allogeneic SCT. In our study, besides the lucarelli class III status at transplantation the only other factor significantly affecting clinical outcome was the day 28 NK cell count. The day 28 NK cell count was noted to be independent of the Lucarelli risk stratification and would serve to complement it as a posttransplant parameter to stratify patient's risk of secondary rejection. Whether interventions based on the day 28 NK cell count would alter the rejection rates remains to be validated in prospective clinical trials.

In summary, this analysis confirms that immune recovery posttransplant in pediatric patients with $\beta$ thalassemia major undergoing a matched related myeloablative allogeneic SCT follows similar recovery kinetics as observed previously with different hematologic disorders [39]. The present study on graft and immune reconstitution characteristics has also helped better define the optimal stem cell dose that one should target, and could not find any clinically significant advantage of using G-CSF primed BM as a stem cell source. The results also suggest that a low day 28 NK cell count increases the risk for secondary graft rejection and might predict death in patients with $\beta$ thalassemia major undergoing a myeloablative matched-related allogeneic SCT. The day 28 NK cell count, a technically easy, highly reproducible and inexpensive assay, could serve as an important posttransplant prognosticator for these patients.

ACKNOWLEDGMENTS

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbmt.2009.01.016.

REFERENCES


Immune reconstitution after HLA mismatched haemopoietic stem-cell transplantation

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Reflection and Reaction

Immune reconstitution after HLA mismatched haemopoietic stem-cell transplantation

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Pill S1470-2045(09)70125-1

As shown by graft-versus-leukaemia effects induced after haemopoietic stem-cell transplantation, the immune system can be used in the control of cancer. [1] During the past three decades, haemopoietic stem-cell transplantation has progressed from being an experimental procedure to a curative option for many patients; [2] and graft-versus-cancer effects have been noted for renal carcinoma, colon carcinoma, and other tumours. [3]

The ideal donors, HLA-identical siblings, are only available for a thir...

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Survivorship issues in hematopoietic stem cell transplantation.

Buchsel PC.

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OBJECTIVES: To review recent research of long-term complications and prevention techniques in hematopoietic stem cell transplantation (HSCT). DATA SOURCES: Peer review journals, books, and research studies. CONCLUSION: Increasing numbers of HSCTs are performed worldwide, leading to an escalating increase in the number of survivors. Only by increased awareness in prevention, diagnosis, and lifelong surveillance can multiorgan toxicities be decreased. IMPLICATIONS FOR NURSING PRACTICE: Community nurses as well as oncology nurses practicing in research and clinical settings have unique access to patients before and after HSCT. Educating and caring for survivors throughout the entire trajectory of stem cell transplantation can assist in establishing best practice techniques to diminish these complications.

PMID: 19411019 [PubMed - indexed for MEDLINE]
Pediatric Cancer Survivorship Research: Experience of the Childhood Cancer Survivor Study


A B S T R A C T

The Childhood Cancer Survivor Study (CCSS) is a comprehensive multicenter study designed to quantify and better understand the effects of pediatric cancer and its treatment on later health, including behavioral and sociodemographic outcomes. The CCSS investigators have published more than 100 articles in the scientific literature related to the study. As with any large cohort study, high standards for methodologic approaches are imperative for valid and generalizable results. In this article we describe methodological issues of study design, exposure assessment, outcome validation, and statistical analysis. Methods for handling missing data, interrater correlation, and competing risks analysis are addressed; each with particular relevance to pediatric cancer survivorship research. Our goal in this article is to provide a resource and reference for other researchers working in the area of long-term cancer survivorship.

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INTRODUCTION

The remarkable successes in treating childhood cancers over the past 40 years have made it imperative to study the long-term outcomes after pediatric cancer and its associated intensive treatments. The Childhood Cancer Survivor Study (CCSS) is one of the first large cohorts of pediatric cancer survivors to be formed and followed successfully. The purpose of this article is to summarize many of the methodological lessons learned over the past 15 years of our experience carrying out survivorship research.1,3 Our intent is to provide a comprehensive document that will prove to be a resource to other researchers in the field.

The topics covered are divided into four main areas. First, we describe the study design and issues involved in contacting and recruiting members of the cohort. This includes the recent changes required as a consequence of the Health Insurance Portability and Accountability Act Privacy Rule (HIPAA). Second, we describe methods for obtaining high quality treatment data that have proven to be vital to the success of CCSS. The third important topic deals with outcome data that are necessarily gathered via self-report mechanisms. For certain outcomes, we have undertaken a validation process to ensure data quality and this is described. Finally, we summarize a number of topics related to statistical analysis of the data, such as handling missing data and competing risks analysis.

MAY DESIGN ANDES OF PAY RECRUITEMNT

Much of the early research conducted on long-term survivors of childhood cancer was carried out as single-institution studies. These early studies documented the occurrence of a variety of late effects of therapy, but were limited in scope due to small number of participants and homogeneity in treatment. Also, because the concept of survivorship was still fairly new, many of these single institution studies only followed participants 5 to 10 years after diagnosis. Early research on survivorship was also performed within the cooperative clinical trials groups. However, because of the therapeutic intent of these protocols, they were not designed for long-term follow-up and often suffered from incomplete participant ascertainment. Thus, much of the early research was restricted to events in the first 5 to 10 years from diagnosis on limited participant populations. Finally, lack of appropriate comparison populations often made interpretation of rates and effect sizes difficult.

The CCSS was designed as a multicenter hospital-based retrospective cohort study with longitudinal follow-up.1 The CCSS has been estimated to have captured approximately 40% to 45% of 5-year survivors diagnosed between 1970 and 1986 in the United States and, in doing so, has established a cohort of sufficient size and heterogeneity to overcome many of these previous limitations.

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The CCSS identified and recruited all survivors meeting eligibility criteria (Table 1) at 26 institutions in the United States (n = 25) and Canada (n = 1). Ascertainment and registration of participants occurred at each center using a comprehensive unified protocol to achieve complete ascertainment of eligible participants. Of the 22,124 participants initially registered with the CCSS Coordinating Center (Memphis, TN), 20,691 were confirmed to be eligible.

Contact began in 1992, and after an initial letter from the treating institution, a letter from the CCSS Coordinating Center containing the baseline survey, informed consent, and a request for medical record release was sent to each eligible patient (or parent if the patient was younger than 18 years at the time of contact). If no response was received, a postal reminder was sent, ultimately followed by a telephone call from the Coordinating Center by a trained telephone interviewer who provided the option of completing the baseline survey by telephone. For eligible participants who were known to have died after achieving 5-year survivorship, their next of kin were contacted and asked to complete the baseline questionnaire. For 7,030 participants unable to be located using the address obtained from the treating institution, a tracing protocol was completed by a national survey research firm (Westat Inc., Rockville, MD). Tracing was successful for 4,188 persons (60%). 

Overall, 3,058 participants (15%) could not be located and were lost to follow-up, 3,205 (15%) declined participation, and 65 participants were unable to participate due to language difficulties. Ultimately, 14,357 eligible participants completed the baseline questionnaire, representing 69% of the total eligible population (approximately six other patients agreed to participate and did so only for subsequent questionnaires). Since the time of the baseline questionnaire, the CCSS has completed three additional follow-up surveys of this cohort, achieving participation rates between 77% and 81% among those participants who were eligible and successfully contacted (Fig 1). In addition, this cohort has been contacted for participation in several other survey-based investigations regarding barriers to health care, sleep and fatigue, use of mammography, health information, and men's and women's specific health issues, in addition to questionnaires specifically targeting quality of life in survivors of bone tumors, and health behaviors in survivors during adolescence.

Comparisons of available demographic and cancer-related characteristics between participants and nonparticipants at the initial base-

![Fig 1. Participation and contact in the Childhood Cancer Survivor Study.](image)

### Table 1. Childhood Cancer Survivor Study Eligibility Criteria

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Diagnosis and initial treatment of leukemia, CNS malignancy (excludes meningioma and craniopharyngioma), Hodgkin's lymphoma, non-Hodgkin's lymphoma, neuroblastoma, soft tissue sarcoma, kidney cancer, or bone cancer (specific ICD-O codes defining eligible cases within each diagnostic category are provided at <a href="http://www.cjsudo.org/ccss">www.cjsudo.org/ccss</a>)</td>
</tr>
<tr>
<td>2. Diagnosis date between January 1, 1970, and December 31, 1986</td>
</tr>
<tr>
<td>3. Age younger than 21 years at diagnosis</td>
</tr>
<tr>
<td>4. Alive 5 years from the date of diagnosis, regardless of disease or treatment status</td>
</tr>
<tr>
<td>5. English or Spanish speaking, because of logistics of questionnaires and interviews</td>
</tr>
<tr>
<td>6. Resident of United States or Canada at the time of initial follow-up contact</td>
</tr>
</tbody>
</table>

relatively small (Table 2). Thus, comparative analyses are always adjusted for these factors.

The CCSS is currently expanding its cohort to include patients diagnosed between 1987 and 1999. This expansion will provide important information regarding late effects of more modern therapeutic protocols, and will employ similar methods of data ascertainment to assure comparability of data with the original cohort. However, a number of challenges to recruitment of this cohort in the current era have been identified. Most importantly, modern privacy laws, including the HIPAA, place limits on contact with eligible participants until their consent for study participation is obtained. In addition, survivors from this era who are age 20 to 39 years are a highly mobile group and not as available or as responsive to contact by traditional mail mechanisms or traditional land-line telephone. Successful recruitment of this population will require innovative use of electronic methods of contact including e-mail and Web-based modalities.

Assessment of therapeutic exposures has been critical to the correct attribution of late outcomes. The CCSS used a methodology of case-by-case chart abstraction for each member of the cohort. Individual abstracters for each center were centrally trained to carry out abstraction of chemotherapy, surgery, and radiotherapy for those consenting cohort members using a standardized medical records abstraction form (MRAF) and treatment data were abstracted from the medical record for each case. In the MRAF the abstractor was asked to specify the dates of therapy covered by that abstraction form, the protocol the patient was treated on (if applicable), and then provide specific data for the treatments of interest (ie, chemotherapy, radiation therapy, and surgery). An individual MRAF form was completed for each treatment plan, but it was recognized that the medical record may not be complete or that some treatments would have been given outside of the participating CCSS centers. In those instances, abstracters were asked to infer doses given for patients and remark on the incompleteness of the dose information so that the incompleteness could be accounted for in the subsequent analyses.

As many pediatric patients were treated on cooperative group studies (Children’s Cancer Group and Pediatric Oncology Group during the treatment era of this cohort) expected doses were calculated for the most common protocols used by both groups. As a quality control measure, abstracted treatment information for each case was compared with the calculated expected doses within each protocol. Outliers were returned to the abstracter to double check the medical records and verify data.

**Chemotherapeutic Agents**

A yes/no evaluation of exposure was asked for each of 42 common chemotherapeutic agents used during this time. For 22 specific agents of the 42, the quantitative dose was abstracted as outlined above. These agents included anthracyclines, alkylating agents, epipodophyllotoxins, and platinum compounds.

For many drugs, the cumulative dose can be used as a measure of total exposure. However, when a number of agents fall into a single class, such as anthracycline or alkylating agents, to enable a succinct assessment of exposure effects for the class, several methods were used. For anthracyclines, the cumulative dose of doxorubicin, daunomycin,
and idarubicin (multiplied by three) were summed. The cumulative platinum compound exposure was calculated by summing the cisplatinum and carboplatinum (carboplatinum divided by four) exposures. Given the wide variety of alkylating agents used, a summary variable was created as follows: First, the dose of each agent was abstracted. Across all patients exposed in the cohort, the dose (standardized by body-surface area) was divided into tertiles of exposure for the individual agent. Each participant was assigned an exposure code of 0 (no exposure), 1, 2, or 3 for each alkylating agent he/she had received. The cumulative score for each individual was summed, and then, across the cohort, these summed exposures were again assigned tertiles. This resulted in individual alkylating agent exposure scores ranging from 0 to 3 for each cohort member which can be utilized in analyses.

**Surgical Procedures**

Surgical procedures were also abstracted and entered into the MRAF. Each procedure requiring general anesthesia was abstracted with the exception of procedures for the placement of vascular access devices. The date, name of procedure, and International Classification of Diseases (9th revision, clinical modification) code were requested for each surgery performed.

**Radiation Dosimetry**

Radiation therapy was also indicated in the MRAF. Abstracters were, however, asked only whether the participant had received radiation, the dates of treatment, and the names of the radiation oncologist and facility where it was given. The abstracters then copied records from the radiation oncology department, including treatment plans, patient placement photographs, daily treatment logs, and radiation summaries; these records were sent to the Radiation Physics Center at the M. D. Anderson Cancer Center (Houston, TX), where the records were scanned and stored in an image database.

The aim was to provide for each patient in a study the radiation absorbed dose to organs or anatomic sites appropriate to the outcome under investigation. Basic treatment information was abstracted for the entire cohort and entered into a database. This first-level abstracting included first and last date of treatment, body region treated, beam energy, treatment field size, configuration and laterality, and total treatment dose. The basic coding is useful for study planning and sufficient for many cohort analyses. Table 3 shows body regions treated for all patients in the database who had radiation therapy, stratified by disease.

Case/control studies and some cohort analyses with specific interest in radiation exposure effects require additional record review, with more detailed coding, in particular where the organ or anatomic site of interest was shielded during treatment (eg, ovaries, testes, breasts, kidneys, or eyes). Dose to the site of interest for each patient is estimated by applying out-of-beam data measured in a water phantom to an age-specific mathematical phantom. Detailed dosimetry is provided for each of these studies, depending on the regions of interest and study population determined by the investigators.

### Validation of Self-Reported Medical Outcomes

Validation of medical outcomes has been an important topic in the CCSS. Due to the increase in the personnel effort and cost required to conduct validation through medical records, however, careful consideration has to be made as to what major end points require this.

---

<table>
<thead>
<tr>
<th>First Malignant Neoplasm</th>
<th>Brain</th>
<th>Head (not brain)</th>
<th>Cranio-Spinal*</th>
<th>Neck</th>
<th>Chest</th>
<th>Spine</th>
<th>Abdomen</th>
<th>Pelvis</th>
<th>Limb</th>
<th>Total-Body Radiation</th>
<th>Region Unknown</th>
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</thead>
<tbody>
<tr>
<td>Leukemia</td>
<td>2,866</td>
<td>91.1</td>
<td>77</td>
<td>2.6</td>
<td>442</td>
<td>15.1</td>
<td>69</td>
<td>2.4</td>
<td>89</td>
<td>3.2</td>
<td>445</td>
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<tr>
<td>(n = 2,927)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>77</td>
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</tr>
<tr>
<td>CNS (n = 1,160)</td>
<td>1,086</td>
<td>94.0</td>
<td>70</td>
<td>6.6</td>
<td>416</td>
<td>35.7</td>
<td>63</td>
<td>5.4</td>
<td>26</td>
<td>2.2</td>
<td>428</td>
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<tr>
<td>Hodgkin's lymphoma</td>
<td>15</td>
<td>4.8</td>
<td>130</td>
<td>12.0</td>
<td></td>
<td>1</td>
<td>0.1</td>
<td>1,484</td>
<td>93.7</td>
<td>1,435</td>
<td>99.6</td>
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<td></td>
<td></td>
<td></td>
<td>0.1</td>
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</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>176</td>
<td>27.9</td>
<td>150</td>
<td>23.5</td>
<td>39</td>
<td>6.1</td>
<td>239</td>
<td>37.4</td>
<td>264</td>
<td>41.3</td>
<td>152</td>
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<tr>
<td>(n = 639)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>7.5</td>
</tr>
<tr>
<td>Kidney tumor (n = 694)</td>
<td>4</td>
<td>0.6</td>
<td>7</td>
<td>1.0</td>
<td>16</td>
<td>2.3</td>
<td>263</td>
<td>37.9</td>
<td>673</td>
<td>97.0</td>
<td>342</td>
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<td>Neuroblastoma (n = 418)</td>
<td>41</td>
<td>9.9</td>
<td>35</td>
<td>8.5</td>
<td>11</td>
<td>2.7</td>
<td>172</td>
<td>42.9</td>
<td>215</td>
<td>51.9</td>
<td>115</td>
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<tr>
<td>Soft tissue tumor (n = 634)</td>
<td>64</td>
<td>8.2</td>
<td>272</td>
<td>39.2</td>
<td>6</td>
<td>0.9</td>
<td>120</td>
<td>17.3</td>
<td>124</td>
<td>17.9</td>
<td>10</td>
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<tr>
<td>Bone tumors (n = 388)</td>
<td>30</td>
<td>7.7</td>
<td>20</td>
<td>5.2</td>
<td>5</td>
<td>1.3</td>
<td>24</td>
<td>6.2</td>
<td>168</td>
<td>43.3</td>
<td>5</td>
</tr>
<tr>
<td>Total [N = 6,606]</td>
<td>4,094</td>
<td>48.1</td>
<td>815</td>
<td>9.0</td>
<td>320</td>
<td>16.8</td>
<td>2,097</td>
<td>24.3</td>
<td>2,564</td>
<td>29.9</td>
<td>940</td>
</tr>
</tbody>
</table>

* Cranio-Spinal added as a category for patients who were treated to the brain and spine. Brain and spine may not have been treated at the same time or to the same dose. Patients with cranio-spinal treatments also are counted in both the "brain" and "spine" categories.
additional effort. At the conception of the CCSS, six primary hypotheses were postulated to be addressed: excess risk of mortality; risk of a therapy-related subsequent cancer; risk of clinically apparent cardiopulmonary events; loss of fertility, adverse pregnancy outcomes, and abnormalities in offspring; distinct patterns of family history of cancer; and increased risk of adverse health events due to health behaviors. Of these hypotheses, the first four listed outcomes were selected for validation in this study.

Vital status and the cause of death were determined through the National Death Index (NDI). There is extensive documentation of the advantages and limitations of the use of the NDI, which is covered in another article in this issue. The remaining three outcomes of interest within CCSS (subsequent cancers, cardiopulmonary outcomes, and adverse pregnancy outcomes) are described in detail herein.

The validation procedure used within CCSS, which has been successful in other large epidemiologic studies, is depicted in Figure 2. Medical outcome data were collected using a self-report survey sent to the home of the eligible participant. A HIPAA form requesting release of medical records was also requested from the participant. On return, a request for photocopies of relevant medical records is then made to the hospital/clinic where the participant was diagnosed for this condition; medical records are reviewed and data coded by trained abstractors/physicians.

Validation through medical records of self-reported medical outcomes from mailed surveys have significant limitations in our current medical care environment. One change to the procedures was brought about by the enactment of the HIPAA Privacy Rule during 2001 and 2002 (modified rule). During recruitment for CCSS, we were able to obtain medical release on 93% of the survivor participants. We subsequently needed to obtain signed HIPAA release for future medical record validation. Although we have ultimately been successful in obtaining these consents for 95% of our participating participants, accomplishing this required significant added resources. Secondly, as these survivors age and become adults, their medical care has transitioned from the pediatric institutions where they were treated for their primary diagnosis to adult care facilities. Because of this transition, and the constant change in health care providers within the current medical system, collection of records from such facilities can be costly and inefficient.

Medical records are useful for identifying false-positive self-reported outcomes; however, it is difficult to identify false-negative outcome events that are not reported by the respondent. In our experience with validation of self-reported outcomes, concordance between self-report and medical records was good for well-known complications that have clear diagnostic criteria, such as the occurrence of subsequent cancers, and for records where the patient had good recollection of where they were seen for the condition, such as pregnancy records and place of delivery. Conditions, however, with nonestablished diagnostic criteria such as cardiac outcomes demonstrated a lower level of agreement and the ability to successfully collect records.

Subsequent Neoplasm Validation

Subsequent neoplasms (SNs) were initially identified by self-report of any relapse or recurrence of their original cancer and/or the occurrence of a new cancer after treatment for their primary malignancy. The name of the hospital where the subsequent cancer had been diagnosed was also requested. All positive responses were screened by a CCSS investigator (J.P.N.), and those responses considered likely or possible SNs were forwarded to the CCSS Pathology Center (Columbus, OH) for verification. Reports of late recurrences of the original cancer (10 years or more after the original diagnosis) were also forwarded for verification. For all positive responses from individuals who signed a medical release, a copy of the pathology report was requested. Returned reports were reviewed by the CCSS pathologist for inclusion or exclusion in the study. Data collected included the specific type of SN, date of diagnosis, and location of tumor(s). If a pathology report could not be obtained, the patient and/or parent response or death certificate and/or other institutional records were reviewed to determine the presence of an SN.

At the time of this report, we had reviewed and verified a total of 2,508 SN events using the above methodology. Among these, 2,196 were verified from the pathology report, and an additional 17 were confirmed from death certificates. The remaining 295 were determined to be valid using participant or proxy responses or other sources as described above. 154 of these neoplasms were in participants who had not signed a medical release.

Adverse Pregnancy Outcomes

To study adverse pregnancy outcomes and possible germine mutations, we evaluated self-reported genetic and congenital diseases among the approximately 6,100 offspring of survivors and the 3,100 offspring of sibling controls. The self-administered questionnaire included questions on pregnancy histories, live births, stillbirths, miscarriages, abortions, cancers, birth defects, and hereditary conditions. Genetic disease included cytogenetic abnormalities, single-gene birth defects, and simple malformations. The approach to validate or confirm the self-reported conditions began with an initial review, including family history, by a cancer geneticist. A decision was made as to whether the self-reported condition could be accepted, rejected, or
that additional information was required. In instances where additional information was required, individualized scripts or questions were prepared for each participant, who was then contacted by CCSS staff to provide additional clarification of the self-report and/or to obtain a medical release for medical records.

All available information on the self-reported condition was then reviewed by a three-person panel to reach a consensus decision. The final decision could be accept, accept but not count because the condition could be explained by family history or nongenetic factors; or reject. Validated genetic and congenital diseases were ascertained in 157 (2.6%) of the children of survivors, compared with 111 (3.6%) of the children of sibling controls. There were no apparent differences in the proportion of offspring with cytogenetic syndromes (seven in case offspring, six in sibling offspring), single-gene defects (14 and eight), or simple malformations (136 and 97). Analyses based only on the self-reported genetic diseases were reassuring and were then confirmed through the validation procedure.

Cardiac Outcome Validation

For survivors who reported a specific cardiopulmonary outcome and were still alive at the time of contact, an additional stage of validation was incorporated which consisted of a series of telephone-based questions (telephone script) to further document the specifics of selected self-reported adverse cardiopulmonary events and to determine the patient's condition another chance for the reported outcomes. Participants contacted by telephone were also asked to sign a HIPAA form and return it to the CCSS Coordinating Center. Once received, medical records were obtained from the physicians listed and were returned to the CCSS Coordinating Center. The first 100 medical records that were returned were reviewed independently by two physicians. Consensus among the physician's validation was reached when two differed and a standardized protocol was developed to come to a consensus of each condition. Subsequent records were reviewed and validated by one physician.

As an example, a flowchart summarizing the validation of 292 survivors who reported congestive heart failure (CHF) is detailed in Figure 3. Among participants for whom validation was successfully carried out, CHF was confirmed for 83% and 67%, and determined to be observed in 11% and 9%, respectively. CHF was confirmed for 83% and 67%, and determined to be observed in 11% and 9%, respectively. CHF was confirmed for 83% and 67%, and determined to be observed in 11% and 9%, respectively. CHF was confirmed for 83% and 67%, and determined to be observed in 11% and 9%, respectively.

Analyses. Instead we have relied on self-reported outcomes for current analyses.

STATISTICAL METHODOLOGY

In the course of analyzing the data from the CCSS over the past 10 or more years, we have needed to carefully consider a number of key statistical issues. Many of these issues are generalizable to other settings, although they have the common theme of being specifically applicable to survivorship research and thus are useful tools for anyone else carrying out statistical analyses on similar data.

Long-Term Survivor Cohort Definition: Impact on Analyses

The requirement that participants have attained 5-year survivorship for eligibility into the CCSS cohort has implications on late events that can be utilized in valid and generalizable analyses. Because our questionnaires are typically worded to ask the first time at which an event occurred, it is not unusual for the first time event to be before the cohort inception time point of 5 years after diagnosis. It is tempting for researchers to examine the rates or carry out time-to-event analyses that incorporate these events. However, caution must be taken since potential patients who died during those first 5 years are not part of the CCSS cohort and have been removed from the denominator; hence,
the full cohort of patients who were at risk for events in the first 5 years are not all included in a survivorship cohort. As such, any time-to-event analyses would not accurately represent true rates or relative risks in that time period and would not be generalizable to any existing prospective population. In statistical terms, this analysis would violate standard principles of time-to-event analyses by conditioning on the future event of survival to 5 years. The most appropriate way of handling time-to-event analyses in a 5-year survivor cohort is thus to begin analyses at the 5-year postdiagnosis time point, only prospectively considering events that occur after the inception of the cohort. One way that outcomes occurring before 5 years could be reported would be as the proportion (or prevalence) of participants who had experienced at least one event by the time the cohort was formed at 5 years. However, for the reasons stated above, one should avoid use of time-dependent rates, or time-to-event analyses. The key point must be emphasized; these results are only generalizable to the population of participants who have survived at least 5 years after their diagnosis of primary cancer.

**Accounting for Correlation Between Survivors and Siblings**

A statistical issue that needs to be addressed in any analysis that incorporates both survivors and siblings is the independence of the outcomes from members of the same family. Since siblings would be expected to have more similar health outcomes than a randomly selected individual from the general population, standard assumptions of independence required for most statistical analyses are violated. In a correlated data setting such as this, unadjusted statistical methods typically lead to incorrect estimation of the variability of measures of association and thus, resulting naïve $P$ values and CIs are also incorrect. To appropriately handle this issue in analyses, a generalized estimating equation approach with robust variance estimates can be used in analyses. These methods have been developed for use with generalized linear models (e.g., logistic and Poisson regression) as well as Cox proportional hazards models. The idea behind the methodology is that it incorporates an appropriate adjustment that accounts for the intraclass correlation and assures that inferences are valid. Other methods for handling the correlation between survivors and siblings that we have used are generalized linear mixed models and bootstrapping approaches.

**Impact of Attained Age on Risk of Disease: Appropriate Methods for Analyses**

The risk of many key outcomes in long-term survivorship studies, especially those of childhood-disease survivors, can be highly dependent on the attained age of the participant and thus, attained age should be incorporated in a meaningful way into analyses. Indeed, if time since diagnosis is used as the time scale for relative risk analyses, rather than age of participant, for example, this can lead to flawed conclusions. In a cohort such as CCSS, participants who enter the cohort between the ages of 5 and 20 years, with 20 years of follow-up, will be age 25 to 40 years at last contact, an age range in which risks of some chronic diseases increase considerably with age. If time since diagnosis were used as the scale for analyses, then participants who were age 25 years would be treated on equal footing with participants who were age 40 years, two groups who might have markedly different risk of disease. As an example, Figure 4 illustrates the difference in expected number of breast cancer cases between three groups of 5,000 participants diagnosed at ages 5, 10, and 15 years, respectively, based on Surveillance, Epidemiology, and End Results (SEER) incidence rates, assuming these participants had the same rates of breast cancer as the general population. Without appropriately taking attained age into account, an analyst might erroneously conclude that girls treated at older ages were more likely to develop breast cancer. One can ameliorate the impact of attained age in descriptive analyses by utilizing standardized incidence rates, adjusted for age. Moreover, for multivariable regression analyses, the use of age as the time scale in a Cox proportional hazards model is an elegant way to directly adjust for changes in risk with age, without needing to incorporate age as a covariate or assuming a specific form for its effect. In this setting, participants enter the analysis at the age at which they enter the cohort and are followed until their attained age at end of follow-up. Another method for analysis is to use Poisson regression models to directly model standardized incidence ratios (SIR) in multivariable models. This method uses external reference age-specific rates such as the rates from SEER to adjust for the effects of attained age on risk of disease. Both these methods provide valid ways of adjusting for the effects of age on outcome and are useful tools for a long-term survivorship data analyst.

**Cumulative Incidence for Nondeath Outcomes**

Most health outcomes of interest are reported using time-to-event analyses and results are often illustrated with figures displaying their cumulative probability over time. Because cohort participants could die any time before that outcome, an analysis of nondeath outcomes must appropriately consider death as a competing risk event when evaluating the probability of these outcomes. Readily available software provides Kaplan-Meier methodology that can be erroneously used in such situations. As described elsewhere, since Kaplan-Meier estimates treat time of death exactly the same way as a censored outcome, the estimates can become overly inflated when many deaths occur during the follow-up period. The appropriate methodology in this setting is to utilize cumulative incidence estimates, which handle deaths differently from censored observations. With the long follow-up period and high mortality rate present in the survivor population, this is an important issue for any analyst to address appropriately in order to obtain valid estimates of cumulative probability.
Missing Data

In any epidemiological study, missing data raise concerns as a potential source of bias. In the CCSS, we have dealt with two types of missing data. The first type arises due to nonparticipation of eligible patients, where all data from the surveys and MRAF could not be obtained. The second type occurs among participants, where some of the survey items or MRAF elements were not answered or collected; this includes survivors who participated in the surveys but did not consent for medical record release.

The first type is difficult to deal with as we have no data on nonparticipants' outcomes and exposures, except their cancer type, age at diagnosis, sex, diagnosis year, and treating institution, which were collected for the initial eligibility establishment. We have compared and reported these characteristics between the participants and nonparticipants, and also made an aggregated-level comparison of MRAF data between the two groups. The CCSS mortality analyses have been an exception. For these analyses, all eligible participants' vital status was ascertained by the public NDI data, and mortality-risk analyses by cancer type, age at diagnosis, sex, and diagnosis year were conducted. When assessing treatment effects on mortality risk, however, we used multiple imputation under the assumption of missing at random of treatment data, given the known characteristics. Note that the multiple-imputation approach imputes missing data multiple times to construct multiple complete datasets, runs an identical analysis with each of the complete datasets, and makes statistical inference using results from the multiple analyses. This is in contrast to the single-imputation approach, where the imputed and observed data are not distinguished in the single analysis of the complete data set; in multiple imputation, the variability across the multiple complete datasets appropriately reflects the uncertainty in the missing data.

For the second type of missing data, the frequencies in the CCSS are mostly no more than 10%, often 0% to 5%. In many CCSS analyses, therefore, we confirm that the extent of missing data in key outcome and exposure variables is small and proceed to perform complete case analyses. When the extent of missing data is large (i.e., the number of incomplete cases is appreciable), or when an adverse event occurrence was indicated in the survey but the age at the occurrence was not reported (thus, a complete-case analysis would bias time-to-event analysis), we used multiple imputation under the assumption of "missing at random." Specifically, we extensively used the multiple-imputation method of Taylor et al with slight modifications for the cases where there were an appreciable number of participants who reported an adverse event of interest, but did not report their age at its first occurrence. This method employs piecewise exponential models to describe the rate of development of each adverse event by relevant demographic, clinical, and treatment variables with possible interactions. Its model fitting uses an expectation-maximization algorithm before proceeding to multiple imputation.

Currently, the Statistical Center (Seattle, WA) of the CCSS is constructing ten complete datasets of CCSS survey participants through an extensive application of multiple imputation so that those who answered the surveys but did not consent medical record release can be entered into analyses of treatment effects. This work involves (1) elicitation of clinical knowledge from pediatric and radiation oncologists on the treatments used from 1970 to 1986 by diagnosis type, age, treating institution, and calendar period; (2) construction of imputation models based on the elicited knowledge as well as statistically driven model selection; (3) imputation of missing data ten times using the models; and (4) checking the imputed data by pediatric and radiation oncologists to see if they are sensible clinically. Such central multiple-imputation of missing data to construct multiple complete datasets has been successfully used in other large epidemiologic studies.

This article summarizes many of the procedures and methodologies implemented in the successful conduct of the CCSS over the past 15 years. These represent our efforts to ensure that conclusions drawn are unbiased and generalizable to the larger population of long-term pediatric cancer survivors. Challenges that require further development of methodology will continue to arise with continued follow-up of the current CCSS cohort and as the more recently treated expansion cohort is incorporated. We already confront issues related to recruiting and maintaining contact with a younger, more mobile cohort. There is an urgent need for recruitment strategies that utilize modern means of contact (e.g., via cell phones and the internet). As we continue to collect data on an aging population, the patterns of missing data will be monitored and documented to assess the need for multiple imputation strategies for additional specific data elements. In addition, the participation rates and demographics of the participating CCSS population will be regularly evaluated. Applying appropriate methodology to the data to adjust for under- or overrepresentation of certain subpopulations will be important if disparities develop. Continued efforts at maintaining high levels of participation will be a priority to reduce any potential biases and to maximize statistical power.

CCSS has been and will continue to be successful at its goals of better understanding and quantifying risks of sequelae to cancer and its treatment. As new knowledge is developed there will be more opportunity for focused interventional studies aimed at reducing the morbidity due to these outcomes. Typically, these will be in the form of screening interventions studies, which can require large numbers of participants to see a significant impact on patient survival or morbidity. This will require the development of efficient study designs, with accurately characterized high risk populations and well defined and meaningful end points that best utilize the available resources and answer the cogent questions.

The author(s) indicated no potential conflicts of interest.
REFERENCES


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The new standard of quality cancer care: integrating the psychosocial aspects in routine cancer from diagnosis through survivorship.

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This article highlights recent findings from the Institute of Medicine of the National Academies of Sciences' evidence-based report Cancer Care for the Whole Patient: Meeting Psychosocial Health Needs published late in 2007. This landmark report lends new credibility to the field and its evidence-based clinical interventions, while outlining a new standard of quality cancer care which mandates that psychosocial aspects must be integrated into routine cancer care. Patients should be screened at their initial visit for psychosocial needs and survivors should have a treatment plan that includes attention to possible increased anxiety on completing treatment, development of posttraumatic stress symptoms, and mixed anxiety and depressive symptoms. Survivors with greater chance of psychosocial sequelae and diminished quality of life are those with chronic physical symptoms, physical impairment, or change in appearance or function. Referrals should be made to proper psychosocial resources (local when possible or by use of the support given by telephone from many site-specific cancer advocacy organizations with telephone help-lines).

PMID: 19060609 [PubMed - indexed for MEDLINE]
Survivorship care: models and programs.

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OBJECTIVES: To review models of care for adult cancer survivors and the challenges in program development. DATA SOURCES: Review of the literature.
CONCLUSION: As the number of cancer survivors continues to grow, so does the need to develop unique evidence-based programs and services for this population. Survivorship should become a distinct phase of care and include: surveillance for recurrence, evaluation of and treatment for medical and psychosocial consequences of treatment, recommendations for screening for new primary cancers, health promotion recommendations, and provision of a written care plan to the patient and other health professionals. IMPLICATIONS FOR NURSING PRACTICE: Many challenges remain to evaluating care models and actualizing clinical services nationally, but oncology nurses are uniquely positioned to take the lead in the care of cancer survivors of all ages.

PMID: 18687266 [PubMed - indexed for MEDLINE]
Pediatric immunohematopoietic stem cell transplantation at a tertiary care center in Cape Town

Lucille Wood, June Juriitz, Jonathan Havermann, Jo Lund, Herman Waldmann, Geoffrey Hale, Peter Jacobse

INTRODUCTION AND STUDY DESIGN: We conducted a retrospective analysis of consecutive referrals of patients under 18 years of age undergoing immunohematopoietic stem cell transplantation to assess the influence of age, diagnosis, graft type and gender on survival. We also contrasted program activity and outcome to that reported from a state hospital in the same geographical area over a comparable period.

METHODS: Conditioning employed either a sequential combination of fractionated 12 Gy whole body and 6 Gy total nodal irradiation separated by 120 mg/kg of cyclophosphamide in patients over 15 years of age. Alternatively, the latter agent was combined initially with oral busulphan and later the intravenous equivalent. Neuroblastoma cases were prepared using a different regimen. In autologous grafted patients underwent ex vivo T-cell depletion with the humanized version of anti-CD 52 monoclonal antibody designated Campath 1H. No additional immunosuppression was given except where matched unrelated volunteer donors were employed.

RESULTS: Sixty-eight procedures were carried out in 61 patients over a 6-year period. Of 11 with acute myeloid leukemia, 8 are alive and well whereas 8 of the 14 with the lymphoblastic variant have died. Of the remaining 12 with hematologic malignancy, all but 2 are alive. Ten of the 17 with aplasia are alive as are all with thalassemia or sickle cell disease. None of the four variables tested affected survival.

CONCLUSION: Our analysis indicates that the standardized preparative regimen, coupled with a new well-established immunosuppressive regimen, is as effective in patients under 18 years of age as in adults. Our analysis also indicates that in a resource-scarce or developing country, it is mandatory to limit high-risk and relatively expensive procedures to active teams that enjoy international accreditation, whether these be in the state or private sector.

Historically, the introduction of classical bone marrow transplantation into this country some thirty years ago centered on adults with problems in the form of rejection and acute and chronic graft-versus-host disease contributing to morbidity and mortality. Despite our early involvement in the use of cyclosporine A as a new generation immunosuppressive agent, these complications continued, although of decreased incidence and severity. The major advance in their reversal was initiation of a series of studies that eventually led to the landmark description of ex vivo T-cell depletion using Campath monoclonal antibodies and our experience continues to accumulate.

Notwithstanding the unquestioned benefits, and in contrast to elsewhere in the world, there remained a substantial local resistance to subjecting children to what was perceived as a still unduly hazardous undertaking! Nevertheless, within 10 years of commencement, it became more generally appreciated that idiopathic and irreversible aplasia could only be cured by allografting whereas a variable response was obtained with antiglobulin globulin and high-dose methylprednisolone. From that time the eligibility for grafting was modified to accommodate younger cases and our initial
results that were reported whilst still at Groote Schuur Hospital.20–22 have recently been updated.23

From January 1995, our established and approved protocols were transferred in to a custom built facility in the private sector. In the new location consecutive procedures continue to be reported to the Center for International Blood and Marrow Transplant Research and this specific team was again re-accredited in January 2003, thereby maintaining a 30-year unbroken record of achievement. Thus, the following analysis from the Department of Haematology and the Bone Marrow Transplantation Unit incorporating The Searll Research Laboratory for Cellular and Molecular Biology at the Constantiaberg Medi-Clinic brings international perspective to outcomes in the pediatric age group undergoing immunohematopoietic grafting in South Africa.

The available facilities, when we pioneered the use of this technology in the Groote Schuur Hospital at the beginning of 1972, were such that management of the young was logistically difficult. This hurdle was overcome by providing separate rooms when the newly dedicated reverse isolation unit was designed and then commissioned in 1982. Given the upgraded physical plant, and expansion of staff to include a social worker, a physician and a psychiatrist with interest in adolescents, it became a realistic option with the first child grafted in 1978. The multi-disciplinary approach was consolidated by participation of Dr Paul Rogers following his return after a period of overseas postgraduate training.24,25

With the relocation of this fully integrated group to an independent academic center it was soon demonstrated, both logistically and functionally, that there was no impediment to providing costly and high technology activities in such an environment.26–28 Continuing outcome analysis from this reverse isolation unit, staffed by nurses trained in all relevant facets with a virtually full-time commitment from pediatricians already in the hospital, rapidly confirmed the feasibility of using advanced protocols approved by Institutional Review Board for conventionally defined indications, in a protected environment dedicated to tertiary hematologic care.17

In the last decade a number of refinements have become standard practice. Among these was the use of matched or minimally mismatched family members and the availability of donations from histocompatible, unrelated volunteers via the South African Marrow Donor Registry.19 To this end, center designation by the European Group for Blood and Marrow Transplantation18 and by the National Marrow Donor Programme followed.29

A priority remains the commitment to whatever innovation is necessary to keep abreast of relevant advances such as the study of chimerism using short term repeat assay.21 This has included exploring the role in thalassemia, sickle cell disease,22 mucopolysaccharidoses,23 common variable immunodeficiency,24 and Fanconi anaemia.25,26 The latter is currently linked to gene therapy being evaluated jointly with Professor Chris Walsh initially in North Carolina,27 and now currently in New York, where special attention has been given to complications from older forms of conditioning that included cyclophosphamide or, even earlier, radiotherapy. Accordingly, this phase has been revised to the use of fludarabine and intravenous busulfan.28

The latter model is to be tested as the basis for contrasting the previously favored myeloablative combinations to primarily immunosuppressive options across the age spectrum.29,30 The rationale for this focus lies in harnessing the formidable capacity of the immune system to identify and eradicate tumor cells and creates a platform for the use of immunotherapy based on subsequent delayed or donor lymphocyte infusions.31 Whether such a theoretically attractive modification will represent a significant advance awaits longer follow up.32

Among the remaining challenges is the problem of mucositis, which impairs nutritional intake, and has led to a focus on the need for developing a structured approach to nasojejunal feeding rather than the more traditional total parenteral approach (Wood, O’Keefe, Jacobs, Dubrovskaya, unpublished). Additionally, there is an increasing appreciation of infections particularly cytomegaloviremia that may progress to organ involvement as in pneumonia and this, together with other infections, possibly related to the use of monoclonal antibodies,33–35 predicated the ongoing study of immunologic reconstitution and viral surveillance with proactive ganciclovir therapy.36

It is against this brief historical outline that outcome in children is presented; the corresponding results in those over 18 years are in preparation (Jacobs, Wood, Juritz, unpublished).

STUDY DESIGN

Our analysis is tabulated for consecutive entries seen in the 6-year period to allow comparison to updated results from the State.13 Additionally, we explored the practicability of operating such a facility, with the specific focus on childhood referrals, outside the confines of the usual base in teaching hospitals. With appropriate attention to financial imperatives and, notwithstanding the ever-constricting focus of changing health care, it
also tests the possibility of maintaining, and continually developing new aspects of this technology in more innovative indications, to keep abreast of worldwide advances.30,31

One modification has been a switch from oral to intravenous busulphan.32 A further logical step will now be the increasing use of fludarabine as a component of mini- or so-called reduced-intensity conditioning regimens that have, as a backbone, sufficient suppression of the immune system primarily to allow host acceptance of the incoming graft. The anticipation is that a graft-versus-leukemia effect will be retained whilst, at the same time, the more serious consequences of the classical myeloablative regimens will decrease.40 Furthermore, in addition to confirming the pattern of hematopoietic reconstitution, a secondary end-point is that of defining immunologic reconstitution.41,42 This endeavor aims to decrease the hazards of infection that notably include those attributable to cytomegalovirus.43,44

METHODS

After recording a detailed history and completing a physical assessment, standard hematologic procedures were employed for a full blood count, aspiration and trephine biopsy.46 The malignancies were classified using conventional cytomorphology supplemented by flow cytometry,47 and cytogenetics48 to precisely delineate each case after which treatment was by risk-stratification to standardized protocols.49-50 The malignancies were classified according to World Health Organization criteria.51 Aplasia was similarly categorized and in those with Fanconi anemia, additional studies included chromosomal fragility after exposure to clastogenic agents.52 Hemoglobinopathy was subdivided by electrophoresis and appropriate biochemical tests.53

For donor selection, histocompatibility testing employed conventional methodology and matching between donor and recipient using international standards via the Hub center of the South African Bone Marrow Donor Programme.59 Donations for matched unrelated volunteers were provided in collaboration with the Anthony Nolan, Australian, German and Leiden Groups as well as The National Marrow Donor Programme.50

All aspects of the treatment including indication, anticipated benefits and potential risks were fully explained by means of extensive counseling after which written informed consent was obtained. Recipients and donors were assessed to ensure suitability for participation and this included clinical examination and non-invasive cardiac and respiratory testing. Placement of a double-lumen catheter54,55 was followed by nursing in reverse isolation.56 Selective decontamination of the bowel was performed with daily fluoroquinolone57 and oral prophylaxis for cytomegalovirus and Pneumocystis carinii, respectively, with acyclovir or valaciclovir58 and co-trimoxazole, or in the case of allergy, an appropriate equivalent.59

Conditioning was 12 Gy fractionated total body irradiation over 3 days at rates of approximately 10xGy per minute, 60 mg/kg of cyclophosphamide over 2 days with intensive hydration, protection of the urothelium with mesna and total nodal irradiation at 1.5 Gy over 2 days.4 The alternative regimen comprised 120 mg/kg of cyclophosphamide combined with 16 mg/kg of oral busulphan,49 or 3.2 mg/kg per day intravenously for 4 days. Patients with Fanconi anemia received cyclophosphamide at reduced doses.51 Irrespective of body weight, a quantitative and qualitatively adequate harvest was obtained after mobilization with stimulatory peptides for 5 consecutive days, by using constantly upgraded apheresis technology on the Cobe Spectra as previously described.60

No prophylaxis was employed against graft-versus-host disease except in those undergoing matched unrelated donations where cyclosporine was maintained at a therapeutic level for 6 months using the C2 assay64 and then tapered to zero over the following half year. Nutrition was a high priority and, whilst intravenous supplementation was not used, there was early recourse to fine-bore nasojejunal tube feeding when needed,65,66 (Wood, O'Keefe, Dubrowsky, Vincent, Jacobs, unpublished).

Comparisons of results between the state and private hospital were made using Pearson's chi-squared tests. Survival time after transplant between groups was assessed using Kaplan-Meier survival curves. Survival times between groups were compared using log-rank tests.

RESULTS

The total number of procedures including retransplants 68 at Constantiaberg Medi-Clinic and 31 at Groote Schuur Hospital. The median age of the patients treated at Constantiaberg was 11.7 years (range 1.7-18.7 years). Hematologic malignancy accounted for 37 cases at Constantiaberg Medi-Clinic (Table 1) and 16 at Groote Schuur Hospital. Benign conditions included aplasia (n=17 at Constantiaberg Medi-Clinic, n=11 at Groote Schuur Hospital) or miscellaneous conditions (n=14 at Constantiaberg Medi-Clinic, n=1 at Groote Schuur Hospital) (Table 2). Apart from the
### Table 1. Patients with hematologic malignancy treated at Constantiaberg Medi-Clinic.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Gender</th>
<th>Interval to transplantation (months)</th>
<th>Age</th>
<th>Type of Graft</th>
<th>Current status</th>
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<td>Alive and disease-free</td>
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</table>

original research report

larger numbers and additional indications in this series, the populations were comparable.13

Day 0 was designated as graft infusion. The median time to reach 0.5 x 10^9/L and 1.0 x 10^9/L neutrophils were 18 days (range, 9-34 days) and 28 days (range, 10-59 days), respectively. The median time for platelets to reach 25 x 10^9/L and 100 x 10^9/L was 17 days (range, 5-32 days) and 27 days (range 13-127 days), respectively.24,26

Transplant-related mortality was death attributable to infection within the first 100 days in the absence of any disease recurrence25 and it accounted for all the patients except for one due to dilated cardiomyopathy. An unusual variant of the acute syndrome of graft-versus-host disease occurred within the first 6 months that was distinctly different from the classical chronic disease.6,26 This was regarded as a forme fruste due to altered immune status consequent upon the ex vivo T-cell deletion,70,71 and occurred in only two cases with successful reversal using topical steroids combined with a short course of oral prednisone.70,73

Cytomegalovirus was demonstrated using the PP 65 assay in two instances that were treated intravenously. One died and in the second no further isolates were present at two weeks although a further 14 days of outpatient oral antiviral treatment followed. Anorexia with nausea and vomiting was readily controlled with seroton and dopamine receptor antagonists although occasionally prochlorperazine was added.24 Diarrhea was assessed with stool culture and treatment of Clostridium difficile when present;25 otherwise simple diarrhea modulation using loperamide hydrochloride, a combination of kaolin with bismuth carbonate, pectin coupled to chlorodyne was used.26 Particular attention has focused on maintaining optimum fluid and electrolyte balance.27 Pain was a major problem when mucositis occurred and initially required oral or parenteral opiates.28 This symptom has been dramatically reduced by the use of sucralfate.29

Hematologic malignancies (Table 1)
Acute myeloid leukemia (11 procedures, 10 patients at Constantiaberg Medi-Clinic) (9 procedures, 8 patients at Groote Schuur Hospital), once disease-free, was treated on this regimen. Nine were allogeneic. One died from cardiomyopathy, a second died in relapse and a third died from recurrence after a second technically successful transplant. Acute lymphoblastic leukemia, most of whom were in second remission (14 procedures, 11 patients at Constantiaberg) (4 procedures, 4 patients at Groote Schuur), were autografted in 9, of whom 5 died in relapse. Of the allografts (n = 2) from siblings 1 died of aspergillosis. A further 3 employed matched unrelated volunteers with 1 dying from multiorgan failure and 1 with recurrent disease. Patients with juvenile myelomonocytic leukemia (1 procedure, 1 patient at Constantiaberg) (1 procedure, 1 patient at Groote Schuur) remain alive. Chronic granulocytic leukemia (6 procedures, 5 patients at Constantiaberg) (2 procedures, 2 patients at Groote Schuur Hospital) was autografted in one who did not achieve cytogenetic remission and after matched unrelated volunteer donation achieved this status by molecular criteria. One syngeneic, three sibling allografts and one further unrelated graft were successful of which one died from cytomegaloviral pneumonitis. The remainder were disease free at the time of writing. Hodgkin (2 procedures, 2 patients at Constantiaberg) (n = 0 at Groote Schuur) and other lymphomas (3 procedures, 3 patients at Constantiaberg) (n = 0 at Groote Schuur) received allografts in 2 and autologous infusion in 3 case. One died from infection and 4 remain in remission.

Benign Diseases (Table 2)
In cases of aplastic anemia (6 procedures, 5 patients at Constantiaberg Medi-Clinic) (10 procedures, 7 patients at Groote Schuur Hospital),8 were allografted and 3 were alive and well at the time of writing. One failed to engraft and despite a repeat procedure died from septicemia. Two received matched unrelated donations: 1 died from septicemia and the other is well. Fanconi anemia cases (11 procedures, 10 patients at Constantiaberg) (5 procedures, 4 patients at Groote Schuur) were all allografted. One required a second attempt and died with septicemia, another developed secondary lymphoproliferative disorder and two died from cytomegaloviral infection and another from multiorgan dysfunction. In this category, damage to the oropharynx and gastrointestinal tract made enteral feeding unreliable and added the costs for limited periods of total parenteral nutrition.8,31 Hemoglobinopathy (6 procedures, 6 patients at Constantiaberg) (n = 0 at Groote Schuur) were all allografted, and were alive and well at the time of writing, although two still had disease present on sensitive biochemical testing.

A miscellaneous group was made up of 1 patient with germ cell tumor that was autografted and died from cytomegaloviral pneumonitis, and 5 patients with neuroblastoma that were autografted and 1 patient died with relapse. A further patient with mucopolysacchari-
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Figure 1. Kaplan-Meier survival for all patients. Kaplan-Meier analysis shows that at 6.8 years there is a stable plateau consistent with cure. It is notable that results are similar for idiopathic aplasia, Fanconi anemia and acute myeloid leukemia with non-significant differences for lymphoblastic leukemia and the remaining cases. There is also no difference in outcome for graft source, but a slight benefit for the female gender, which did not attain statistical significance.

Overall Survival
Overall survival was 60% at 7 years of follow-up (n=59 at Constantiaberg Medi-Clinic) (Figure 1). Notably, survival was similar for idiopathic aplasia, Fanconi anemia and acute myeloid leukemia with non-significant differences for lymphoblastic leukemia and the remaining cases. There is also no difference in outcome for graft source, but a slight benefit for the female gender, which did not attain statistical significance. Given the small numbers it is not surprising that none of the variables achieved significance. Quality of life studies\textsuperscript{223} were not formally carried out in this investigation.

DISCUSSION
These results are comparable to published first world outcomes. Our analysis allows four conclusions. Firstly, the scientific and ethical aspects of immunohematopoietic stem cell transplantation, whether the graft is derived from bone marrow or peripheral blood, when conducted by an appropriately constituted and functioning multidisciplinary team, operating in a private academic center, demonstrably meets worldwide standards of practice. This is reflected in satisfactory audit with ongoing re-accreditation of this particular team by the International and Autologous Bone Marrow Transplant Registries now combined as the Centre for International Blood and Marrow Transplant Research with endorsement as a transplant, harvest and donor center by the European Group for Blood and Bone Marrow Transplantation as well as for performing these procedures as designated by the North American National Marrow Donor Program.

Secondly, while indications continue to undergo redefinition, correct case selection is imperative, if the best needs of the community are to be served. Thus, access to the technology must be widely available and, by definition, this includes responsible resource allocation between state and private sectors. To this end it is constructive to contrast the present experience to our initial reports\textsuperscript{40-41} that were recently updated from a state hospital.\textsuperscript{19} It can be seen that there is no substantial difference in demography, procedure or results other than, in the current series, somewhat larger numbers, wider indications, use of intravenous busulphan and inclusion of matched unrelated volunteer donors. Of relevance is that, at least in siblings, further immunosuppression is not needed after ex vivo T-cell depletion and this translates to financial cost saving with possibly a decrease in morbidity. It therefore follows that even when alternative graft sources are used, the relatively high initial costs are clearly offset by the potential for cure and so justify the maintenance and indeed ongoing development of such a therapeutic option. This situation differs significantly from regimens using unmanipulated grafts which would then typically include cyclosporine A, short-course methotrexate and often corticosteroids as necessary to control rejection and blunt acute as well as chronic graft-versus-host disease. To accommodate these variables identification of teams that fully and consistently meet standards, of which the center-effect necessitating a minimal number of procedures to comply with agreed activity, is mandatory to prevent this important treatment option becoming discredited as a result of inadequate performance. Whether a mechanism for national scrutiny and registration can be established is currently conceivable but whether it would ever become acceptable in the wider world remains moot. Currently at least compliance with the approved guidelines from The North American Foundation for the Accreditation of Hematopoietic Stem Cell Therapy\textsuperscript{24} as well as the Joint Accreditation Committee of the International Society for Hematopoietic Therapy and Graft Engineering in Europe and the European Group for Blood and Marrow Transplantation\textsuperscript{4} would be a desirable starting point. In this context there is also the crucial balance between

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the undoubted need and funding provided by managed health care required to allow private sector programs to match state activities with the latter having a reciprocal obligation to provide for individuals with limited resources. In these circumstances utilization of these procedures should be shared on the basis of resource availability for individual patient groups.

Thirdly is the crucial caveat that only peer-reviewed protocols, having undergone scrutiny by Ethics and Research Committees coupled with institutional Review Board endorsement, are supported. These need, in a single center by definition, to have the expertise and flexibility to accommodate appropriate patient categories and to do so irrespective of age. Given the constant revision of the role that such interventions generate an acute awareness of shifting entry indications is vital. For example there is now appropriate and decreasing referral in acute myeloid leukemia when the presence of specific, or good risk, cytogenetic markers show equal outcome with optimum chemotherapy. Furthermore, the competing role of small molecules such as retinoic acid and arsenic in progranulocytic tumors on the one hand and signal transduction inhibition in chronic granulocytic leukemia using imatinib on the other, alter the previously automatic inclusion of stem cell transplantation in treatment algorithms. There needs to be a consciousness that these expensive but potentially curative interventions retain a role in Hodgkin and other lymphomas, but outside clinical trials, preferably international, raise serious reservations about uncritical and random usage. Concerns focus on small numbers of cases in unregistered practice by occasional therapists not reporting results. Implicit in such ongoing scrutiny is an obligation for unit or team recognition based upon outcome analysis and peer review accountability raising the spectre of there being some sensible restriction to preferred providers or designated units. These need to be defined by published survival data and, most certainly, not the alternative, but convenient choice of only cost as the defining factor for so-called preferred providers.

Fourthly, among the areas of expanding interest are the solid tumors. The present data are too small for interpretation and only establish technical feasibility. The continuation of this commitment, perhaps more so than in conventional usage, should not be restricted but rather encouraged, within the context of thoughtful, registered and multicenter trials.

CONCLUSION
This interim analysis of ongoing studies presents outcomes consistent with international standards of practice and supports the appropriateness of continuing to provide immunohematopoietic stem cell transplantation to properly selected cases throughout South Africa. This sub-Saharan stance could serve as a model for other under-resourced countries worldwide. Predictably no difference in results could be found between those from this private academic center or a state hospital emphasizing that allocation of transplantation should be shared as dictated by availability of resources in each sector. Furthermore, since these are uniform irrespective of age, separate pediatric units are difficult to justify in the face of a limited and ever deteriorating economic climate. Whilst acknowledging substantial financial costs and morbidity and mortality, these are offset by the curative potential of this form of treatment and, accordingly, they remain an obligation, but should be provided, and fully supported, only in those centers that meet stringent international criteria, including regular audit, and consequently enjoy ongoing accreditation by major registries worldwide.

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original research report

**Immunohematopoietic stem cell transplantation in Cape Town: a ten-year outcome analysis in adults**

Lucille Wood, Jonathan Haveman, June Juritz, Herman Waldmann, Geoffrey Hale, Peter Jacobs

*BACKGROUND AND OBJECTIVES:* Immunohematopoietic stem cell transplantation has curative potential in selected hematologic disorders. Stem cell transplantation was introduced into South Africa in 1970 as a structured experimental and clinical program. In this report, we summarize the demography and outcome by disease category, gender, and type of procedure in patients older than 18 years of age who were seen from April 1995 to December 2002.

*PATIENTS AND METHODS:* This retrospective analysis included 247 individuals over 18 years of age for whom complete data were available. These patients received grafts mostly from peripheral blood with the appropriate stem cell population recovered by apheresis.

*RESULTS:* Patient ages ranged from 20 to 65 years with a median age of 42 years. There were 101 females and 146 males. There were no withdrawals and 63% survived to the end of the study. At 96 months of follow-up, a stable plateau was reached for each disease category. Median survival was 3.3 years (n=26, 14.6%) for acute lymphoblastic anemia, 3.1 years (n=44, 18%) for acute myeloid leukemia, 2.8 years (n=47, 19%) for chronic granulocytic leukemia, 2.8 years (n=21, 29%) for lymphoma, 1.5 years (n=23, 9%) for myeloma, 1.43 years (n=10, 4%) for aplasia, and 1.4 years (n=38, 15%) for a miscellaneous group comprising less than 10 examples each. Multivariate analysis showed that only diagnosis and age had a significant impact on survival, but these two variables might be interrelated. There was no significant difference in outcome by source of graft.

*CONCLUSION:* The results confirm that procedures carried out in a properly constituted and dedicated unit, which meets established criteria and strictly observes treatment protocols, generate results comparable to those in a First World referral center. Low rates of transplant-related mortality, rejection and graft-versus-host disease are confirmed, but the benefits cannot be extrapolated outside of academically oriented and supervised facilities.

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**Traditional allogeneic bone marrow transplantation, introduced primarily for correction of irreversible aplasia whether congenital or acquired, may also be life saving in other defects exemplified by immunodeficiency disorders and metabolic diseases.** It is currently accepted as having a place in treating acute1 and chronic leukemia,1 Hodgkin’s or indolent2 as well as aggressive lymphoma3 in addition to myeloma. The scope increasingly extends to a number of solid tumours amongst which are breast cancer4 and, in selected cases, acquired lesions including paroxysmal nocturnal haemoglobinuria3 or myeloproliferative syndromes amongst which is idiopathic myelofibrosis.13

Outcome continues to benefit from more precise matching at both major4 and minor13 histocompatibility loci, using DNA-based or molecular techniques, which also make possible definition of chimerism16 or quantification of minimal residual disease.17 Nevertheless problems persist amongst which are rejection18 and acute15 or chronic graft-versus-host disease.20 These are offset by better utilisation of immunosuppressive regimens for conditioning21 as well as in the post-procedure period with corticosteroids, methotrexate,22 cyclosporin,21 tacrolimus24 and mycophenolate mofetil.28 Concurrently
there is greater attention being given to supportive care, with proactive evaluation and maintenance of nutrition underpinned by strong psychosocial networks. Against the background of this therapeutic option was introduced into South Africa in 1970 via an animal allotransplant graft program with active research and ongoing clinical development sustained by the original international team. On this basis it was possible to provide a forty-year perspective in which four separate developmental, albeit overlapping, periods can be identified with the appreciation that such distinction is somewhat artificial.

Firstly was a need to pioneer the use of apheresis in this country so replacing the obstructed delivery of blood products in bottles and, applied initially, to explore a place for granulocyte transfusions. There was subsequent permeation of the technology into commercial blood banks where, in the course of the last 30 years, it has entirely appropriately become a well-established routine. Concomitantly to expand the use in processing bone marrow after collection whilst, latterly, to directly harvest the corresponding population after mobilisation with stimulatory peptides into the peripheral blood. The latter resulted in a substantially shortened time to engraftment, a low incidence of relapse in acute myeloid leukemia, but reappearance of cytomegaloviral infections.

Secondly were preliminary cooperative studies with Jeanne Borel at Sandoz in Basel showing that cyclosporin A, at least in experimental animals, decreased both the incidence and severity of acute graft-versus-host disease. Follow-up in the clinic, however, revealed that while these benefits could be reproduced in patients, prevention was only partial and did not abrogate recurrence.

Thirdly there followed collaborative research initially in Cambridge and with the same group continuing these investigations latterly in Oxford that defined a role for T-lymphocyte depletion using Campath monoclonal antibodies in vivo by adding them to the graft in the bag. One consequence was recognition of an altered expression or forme fruste of immunologically mediated acute graft-versus-host disease that appeared later, was typically limited to skin and usually responsive to topical steroids although on occasions systemic administration was unavoidable.

This syndrome is not considered chronic in the accepted sense either to time of onset or more importantly having a quite different clinical spectrum and remains distinct being conceptually regarded as reflecting modulation by the antibody used in this particular way. Such an approach remains firmly established as local, and much wider, practice. It also forms part of the broader ongoing participation and reporting of results to the Campath users group. The original lytic IgM protein was subsequently replaced by an epoic equivalent and more recently shifted to a chimeric humanised version designated Campath 1H. In all these studies engraftment remains uniform and rejection infrequent. This applies even when extended to include family members and matched unrelated volunteer donors identified through the South African Bone Marrow Registry interacting with corresponding European Centers and the American National Donor Program.

Fourthly, appreciating that peer review was crucial to maintaining standards, but unfortunately non-existent in this country or anywhere in sub-Saharan Africa, every patient, from inception, has been reported to the International and then Autologous Bone Marrow Transplant Registries for scrutiny and audit. After more than twenty years of refinement and consolidation, our original program was transferred, in 1995, to an academic Department in the private sector. Maintenance of this active surveillance mechanism, now to the Center for International Blood and Bone Marrow Transplant Research, has sustained unbroken reaccreditation to the present time. Concurrently the original group remain designated as a Transplant, Harvest and Donor Center within European Bone Marrow Transplant Registry and concurrently with participation and approval for transplant placement as well as and as harvesting by the American National Donor Program.

After 30 years it is appropriate that this single team experience be updated to allow comparison, particularly in the last decade, to other private and state services in this country, but, more critically, to be measured against results on a worldwide basis.

It is mandatory in South Africa, as in other emerging, developing or under-resourced areas, that a clear and reliable statement of what can be expected is available. Such statistically analysed data provides perspective against which to evaluate changes that continue to occur as a result of the ever-shifting landscape of managed care for the insured patients. This is especially relevant as government seeks to focus on costs in private hospitals whilst medical aids seek to define preferred providers, not as one would expect on cost-effective benefits, but primarily using financial or incentive-driven criteria. Scientifically, academically and intellectually, it is argued that such monetary focus is inappropriate and should defer to the identification of high-performing multidisciplinary units that can meet the requirements for the widely acknowledged center effects. Corresponding information for those under 18 years of age is the subject of a separate publication.

To definitely document the introduction and subse-
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quent development the comprehensive experience in this country has recently been reviewed.48

Also the wider place of these interventions from introduction at the University of Cape Town and Groote Schuur Hospital in 1970 with establishment in the private sector with continued development over the last 15 years is in preparation for the History of Medicine Series.49

Accordingly, we detail, from this academic unit in Cape Town, statistically analysed results and define projected basic as well as clinical research that will focus on immunologic reconstitution.50 The latter is anticipated to correlate laboratory findings with patient response to viral, parasitic and bacterial infections in the context of reverse isolation and emerging multi-drug resistance.51

The particular relevance is that this program will explore immunosuppression by only ex vivo T-cell depletion that is distinctly different from many more conventional approaches where a range of agents are administered for periods of time after engraftment for control of rejection and graft versus host disease. Additionally, so better document commitment to quality-of-life assessment with psychosocial counselling in conjunction with liaison psychiatry. There already emerges a strong argument to restrict these costly treatments to active teams that have a clear record of meeting first world performance judged by published outcome, attract sufficient volume to sustain the advantages of the well-recognised center-effect.52-53 and, in developing or under-resourced areas, meet criteria for international acceptance via endorsement as full participating membership in appropriate registries.54 Indeed it has recently been proposed that these activities be collected and analysed by establishing a repository within the South African Bone Marrow Registry to match the ongoing survey by European Bone Marrow Transplant Group.55 The latter focuses on cord blood but draws attention to the widely recognised argument to require reporting of all procedures while leaving the way open for local groups to assign resources regionally. These are not exclusive but can readily be revised to include survival analysis in the future.

METHODS

Diagnosis

Following comprehensive clinical assessment agreed definitions were used for aplasia, acute and chronic leukemia, Hodgkin's or other lymphomas and myeloma.56 Morphologic features on bone marrow aspiration were supplemented by appropriate cytochemistry,57 flow cytometry,58 karyotyping and molecular genetics documented.59 Immunohistochemistry was used as necessary on tissue and trephine biopsies.59

Radiology

Conventional films were complemented by computerized axial tomography including determination of bone mineral density.60 Skeletal involvement was recorded and changes graded according to locally developed criteria (unpublished). The preferred PET-CT was not routinely available during this time.

Biochemistry

Renal and hepatic profiles, serum protein electrophoresis, immunoglobulin quantitation and markers of tumour activity included sensitive C-reactive protein and S2 microglobulin.

Staging

Where relevant this was according to the Cotswold modification of the Rye system or, preferably, the international prognostic index.61

Pre-transplant phase

Physical facility

Management was in reverse isolation where each two-roomed suite had dedicated shower and toilet. There was a particular focus on patient information and interactive counselling offered to help maintain optimum quality of life.62-63 In addition details of local bone marrow transplant unit were provided in two specially written information brochures.64-65

Nutritional status

Selective decontamination of the bowel relied on oral levofloxacin.66 A low microbial diet was provided with daily review of weight, caloric status and vitamin as well as trace metal balance. All patients were in the routine care of a trained academic dietician.

Where targets were not achieved placement of a nasojejunal, as opposed to nasogastric, fine bore feeding tube was used and this remains a subject of evaluation (Wood, Schloss, O'Keefe, Jacobs - study in progress).

PATIENTS

Three hundred and twenty consecutive individuals, over 18 years of age, eligible for immunohematopoietic stem cell transplantation were registered from April 1993 to December 2002, a number of which were included in a recent audit by the Center for International Blood and Marrow Transplant Research and retrospectively analysed. After the data was cleaned to implement age restriction a total of 247 cases qualified as the basis for this report.
Total parenteral nutrition was infrequently needed and then largely in the paediatric age group.

Nausea and vomiting
These symptoms were anticipated and recipients received 72 hours of oral phenobarbitone, metoclopramide or serotonin antagonists prior to admission. This was switched to intravenous route at the time of conditioning and dosage titrated to proactively prevent such side effects with this regimen supplemented by paracetamol, dexamethasone or valoid orally or by suppository as needed. More recently neurokinin-1 antagonists and second-generation 5-hydroxytryptamine antagonists have become available and, together with motility-controlling drugs, were used when indicated. Clostridium difficile was treated with oral metronidazole or, if resistant, vancomycin by the same route.

Neutropenic fever and viral infections
Pyrexia, defined as 38°C, when confirmed at 60 minutes, required repeated cultures of blood, urine and stool followed by empiric single agent ceftazidime or tazocin with the beta-lactams given as 24-hour continuous intravenous infusion. Isolation of pathogens was the basis of switching to in vitro sensitivity directed regimens and typically with 2 drugs synergistic in vitro for persisting fever.

Culture-negative cases were managed with 1 mg/kg of amphotericin in 200 ml 5% dextrose water given, as a continuous 24-hour infusion since side effects, and particularly nephrotoxicity, were abrogated. Cyto megalovirus was monitored by serology and more recently routinely screened for by the PP65 and PP67 assay with polymerase chain reaction when leukocyte counts were below 1x10^9/L. Pre-emptive treatment was by intravenous gancyclovir or valganciclovir and switched to the latter agent orally for a further two weeks once the virus was no longer detectable. It is notable that, at least in solid organ transplants related to the pharmacokinetics and pharmacodynamics, both agents can result in drop in white count and delay recovery in neutrophil and monocyte levels.

Transfusion policy
Neutropenia, particularly when associated with fever, of less than 0.5x10^9/L granulocytes received 300g GCSF intravenously until this level was stable above 1x10^9/L and temperature again normal. Packed red cells were administered on a standard regimen for symptom-relief with haemoglobin arbitrarily kept above 100 g/L. Platelets were used in those at risk from bleeding to maintain counts greater than 30x10^9/L with single-donor apheresis units concurrently with 500 mg of cydokapron orally or intravenously every 8 hours. These were collected initially from a dedicated volunteer panel and with quality control to meet safety standards.

Surveillance
Data analysis was on a moment-to-moment basis. All decisions were updated daily at morning report and included planning for nutrition, psychosocial counselling, liaison psychiatry, physiotherapy with review of medication by haematology pharmacists.

Central venous access
This was secured using a double-lumen Brovic catheter inserted under general anaesthetic following the Hickman technique. The position was checked on recovery to confirm that return of muscle tone did not dislocate the tip, ideally positioned 1 cm above the junction of the superior vena cava with the right atrium. Low dose warfarin, with the INR in the normal range, was employed to reduce the risk of venous thromboembolism.

Counselling and liaison psychiatry
At diagnosis a family conference was convened that included introduction to nursing and paramedical staff, dietician and physiotherapist. Previous cytotoxic drug treatment and irradiation was documented and the risks, as well as potential benefits, of the procedure explained and brochures describing the organisation of the department and the unit provided. Once all issues had been fully discussed the informed consent was signed by patient or, in the case of minors, by parents or responsible guardians.

Prior treatment protocols

Donor selection
Histocompatibility was confirmed by standard methods via the South African Bone Marrow Registry where appropriate searches extended to include British, Australian, Dutch and American participating centres; further consultation was with the Leiden Group. These activities were part of a worldwide research project with certification through the International Immunogenetics Group.

Acute lymphoblastic leukemia (n=14: 6%)
Treatment was standardised to the Berlin-Frankfurt-Munster or BFM programs.
**Acute myeloid leukemia (n=44; 18%)**

Initially the combination of cytosine arabinoside, an anthracycline antibiotic with an epipodophyllotoxin, was used. More recently this was extended to the British Medical Research Council AML 15 program. In some instances managed health care organisations, on advice of their local consultants, have unfortunately restricted access to these regimens despite clear evidence of benefit from adding gemtuzumab ozogamicin to this drug regimen.

**Chronic granulocytic leukemia (n=47; 19%)**

Disease control was rapidly achieved using hydroxyurea and latterly imatinib mesylate. Transplantation was offered to fully matched recipients particularly with the escape from the tyrosine kinase, if cytogenetic abnormalities developed or this was patient’s choice.

**Hodgkin and other lymphomas (n=71; 29%)**

Current protocols parallel those used by the European Organization for Research and Treatment in Cancer (EORTC) or the German Hodgkin and Lymphoma Study Group.

**Myeloma (n=23; 9%)**

Stratification was by comorbidity and risk factors using guidelines from the International Myeloma Working Group. VECD comprising vincristine, epirubicin, cyclophosphamide and dexamethasone were preferred. Here the target was greater than seventy-five percent reduction in both paraprotein level and plasma cell infiltrate of marrow trephine biopsy to qualify for autologous immunohaematopoietic stem cell grafting. Thalidomide was not approved by local third party funders during these studies. In appropriate cases maintenance depended on negotiating for this agent with bortezomib still not being available. Alternatives were pulsed melphalan and methylprednisolone or salvage with dexamethasone and vincristine.

**Aplasia (n=10; 4%)**

Intensive support included limiting blood and other components as far as possible. Where suitable sibling donors were available this remained the preferred treatment. In other instances immunosuppressive regimens including high dose methylprednisolone and antilymphocyte globulin were standard.

In keeping with established practice, including this country, irreversible bone marrow failure was a prime indication for one or other form of immunohaematopoietic stem cell allografting either matched sibling, unrelated or minimally mismatched family donors.

**Miscellaneous group (n=38; 15%)**

This varied by subtype. In hairy cell leukemia 2-chlorodeoxyadenosine was largely successful and so seldom needed grafting. Cutaneous lymphomas, including mycosis fungoides or Sézary syndrome, received appropriate superficial irradiation. Gemcitabine or alemtuzumab for tumours of T-lineage and individualised therapy in B-lymphocyte neoplasms appropriate for risk category. Individuals received pulsed chlorambucil or combinations of rituximab, fludarabine and cyclophosphamide; latterly the anti-CD20 monoclonal antibody Campath or alemtuzumab. In selected refractory instances allografting was an option.

**Special projects**

A number of prospective investigations are actively evaluating changes in pulmonary, skeletal, gastrointestinal, renal and cardiovascular status as an integral part of management and incurred new costs. Immunologic reconstitution is to become a major focus of the program to explore the importance of side effects better now described as survivorship.

**Transplantation phase: conditioning regimens**

Two myeloablative options were used. In none of these patients was this primarily immunosuppressive-alternatively described as reduced intensity.

**Radiotherapy**

12 gray fractionated whole body irradiation on days -7/-6/-5 is followed by 60 mg/kg of cyclophosphamide intravenously on days -4 and -3, and 6 gray fractionated total nodal irradiation on days -2 and -1. The graft is infused on day 0.
Chemotherapy
Bisulfan combined with cyclophosphamide, used originally in the four-day regimen has been replaced by two-day alternative: the former being intravenous as opposed to oral.\textsuperscript{134,135} In selected individuals, where this agent was contraindicated, fludarabine was used. Additionally in over eighty percent of our cases the first choice was the BEAM preparative regimen.\textsuperscript{136}

Re-transplantation
With primary or secondary graft failure preparation was with fludarabine\textsuperscript{137} antilymphocyte globulin and cyclophosphamide.\textsuperscript{138}

Mobilization and quality control
Granulocyte-colony stimulating factor was commenced subcutaneously on Day 5 at a flat dose of 300\textsuperscript{132}mg with the last injection at 04h00 on the day of first large volume apheresis harvest. CD34 population were noted but not specifically used to time these collections.\textsuperscript{12}

Autografts
Cyropreservation was undertaken as previously described.\textsuperscript{139}

Allogeneic transplants
Histocompatible siblings received only the ex vivo T-cell depleted product after exposure to Campath 1H in-the-bag.\textsuperscript{130} Alternative family members and matched unrelated volunteer donors - exclusively - received cyclosporin at full therapeutic dose for six months with a fifty percent reduction at three months and the remaining three months at twenty five percent optimum dose.

Infusion techniques
Premedication, given half an hour before the graft containing the monoclonal antibody, consisted of 100 mg of hydrocortisone, 12.5 mg of promethazine hydrochloride intravenously and 500 mg paracetamol orally. Continuing non-invasive cardiovascular and respiratory monitoring was sustained until vital signs were stable. Oxygen desaturation necessitated rate adjustment of this infusion and this occurred in less than five percent of the procedures. In approximately thirty percent pyrogenic reactions with abdominal discomfort were attributed to the immunoglobulin. There were no lasting side effects. In autografts transient fever was initially attributed to presence of DMSO and recently ascribed to contaminating granulocytes in the apheresis product.\textsuperscript{140}

Product manipulation
Bone marrow, although no longer routinely used, continued to accommodate matched unrelated volunteer programs predicated on requirements of the coordinating center from different parts of the world. Here the harvest was modified using the standard apheresis technology: vide supra.\textsuperscript{139} once greater than ninety-five percent of the mononuclear cells have been recovered the residual blood was infused to the donor or discarded. The immunohaematopoietic stem and progenitor concentrate had the standard ex vivo addition of 20 mg of Campath 1H, incubated at 37° for half an hour and infused.\textsuperscript{141}
Post-transplant surveillance
Immunosuppression was not used in histocompatible siblings. Conversely, in family members and matched unrelated donors, the recipients received cyclosporin maintaining therapeutic levels of 1500 ng/mL on the C2 assay. In the dose was cut to fifty percent at six months, twenty five percent at nine months and discontinued at twelve months protocol included 200 mL of stabilised human serum weekly in addition to 500 mg of valaciclovir twice daily for viral prophylaxis for three months and co-trimoxazole comprising trimethoprim 80 mg and co-trimoxazole 400 mg daily for one year.

Statistical analysis
Data was examined using the Kaplan-Meier product limited estimator, Cox proportional hazard based on initially fitting each risk factor, multivariate analysis and Pearson Chi squared contingency tables for independence testing.

RESULTS
Age and gender
Median and mean age were both 42 years with a range from 19.45 to 65.0 (Figure 1). At transplantation, mean age for females was 41.3 years and for males 42.7 years. Most were between 40 and 50 years with a much-reduced number between 60 and 70. There were 101 female and 146 males. Gender was balanced in the middle but at the other end of the spectrum roughly double the number of males were present. Survival decreased with age (Figure 2) but despite difference between the groups, it is notable that the estimate of survival function is roughly the same. This suggests that once the initial transplant-related mortality is past, there is equal benefit from the procedure. There are no gender differences evident (Figure 3).

Influence of transplantation procedure
When subdivided by those having peripheral blood stem cell autologous or allogeneic transplants and further into groups were the graft source was from bone marrow or peripheral blood treated respectively with the of opsonic monoclonal antibody Campath IG or the humanised variant designated IH survival appears unaffected by the immunoglobulin (Figure 4). There was no statistical difference between control patients receiving peripheral blood stem cell autografts (n=101) when ex vivo t-cell depletion is carried out with the Campath IG bone marrow (n=14) or blood (n=36). Similarly, when humanized monoclonal antibody IH was used in-the-bag on the mononuclear
cells driven by apheresis from the circulation (n=96) outcome was comparable.

Outcome by diagnosis
The majority of the patients were referred with non-Hodgkin lymphoma followed by chronic granulocytic and acute myeloid leukemia (Figure 5).

Analysis of survival on this basis (Figure 6) (Table 1) shows substantial differences with superiority for the acute leukemias followed by lymphoma although these are not stratified for Hodgkin and other variants with approximately equal outcome for the remaining cases with aplasia and myeloma.

Cause of death
The majority were multifactorial having a final common pathway of severe inflammatory response syndrome culminating in multiple organ dysfunction. Where separation and assignment to particular predominant explanation was possible, relapse, followed by respiratory failure accounted for most of the remainder with small numbers probably artificially attributed to sepsicaemia and cerebral events (Figure 7).

DISCUSSION
The introduction initially as conventional transplantation of bone marrow and subsequent development in South Africa over the ensuing 35 years has, in the last decade, started to raise questions about sustainability of these often life saving, albeit costly, endeavours. Unquestionably they require a substantial investment of resources in providing tertiary or even quaternary level physical facilities originally based in state supported University teaching units but increasingly re-deployed at privately funded hospitals or clinics. Both areas depend for continued effective function on retaining a stable complement of highly trained and experienced doctors with nurses as well as paramedicals that make up the indispensable multidisciplinary team. Here arises the first problem, and it is as acute as serious, with severe understaffing becoming ever more critical at all levels reflecting a steadily accelerating exodus from South Africa of these professionals. Secondly, in monetary terms, there is inevitable competition with other health care priorities most strikingly in sub-Sahara by those created in the wake of an escalating acquired immunodeficiency disease pandemic.

Sharp focus is given to these sobering realities by an analysis describing such activities in Europe coupled with a recent survey for the first time documenting the current local situation. Accordingly the time was judged appropriate to set out the way in which the latter point had been reached with a further intention of offering perspective for the future of immunohaematopoietic stem cell grafting in a Third World country seeking to objectively balance need against capacity. Such a repository has a number of potential benefits. It will allow government or other licensing authorities to look at the way regional activities are distributed. Secondly to ensure equitable accessibility to state funded programs and for all centers, particularly those in the private sector, to show effectiveness in adults and children, determined objectively by outcome analysis. Such impartiality facilitates scrutiny by managed healthcare organisations and medical aids of results from individual teams and so shifts the responsibility...
Stroke
Matched unrelated donor
Multi organ failure
Others
Rejection
Respiratory failure
Septicemia
Transformed

Figure 7. Causes of death.

It is crucial to ensuring that potentially incentive-driven financing is replaced by supporting preferred providers where audit and accountability take into consideration the all-important center effect.

In an endeavor to provide this type of non-partisan information consecutive admissions over a seven-year period were analyzed from an academic center based in the private sector. The facility was specifically created to recapitulate the standard University style department. Interestingly, the same investigators that designed and commissioned the prototype and custom-built bone marrow transplant unit in the Groote Schuur Hospital have extended this experience over 35 years.46

Thus with the relocation of the Jean Porter apheresis unit and many of the professional nurses it has been possible to continue reporting consecutive recipients to the Center for International Bone Marrow Transplantation Research. This has ensured that their scheduled audits continue with accreditation: a status, which has remained intact for more than three decades.

Using this database the paediatric results could be compared to earlier experience from the State Hospital and document, apart from a more active private program, essentially the same results.47

The parallel experience in our adult cohort supports five conclusions.

Firstly, given a suitable physical facility and adherence to management protocols that are commensurate with international standards of care, an environment could be created to meet and then sustain established criteria for international standards of care whilst continuing to develop and introduce new research topics as appropriate. The cardinal consideration was a focus on the all-important haematology co-ordina-
tor managing a multidisciplinary management team made up of long stay nursing and paramedical professionals including academic dieticians and skilled janitorial support staff with constant interaction between infectious disease, cardiology, nephrology and gastroenterology consultants. Such a comprehensive unit emerges as the indispensable key to provision of unquestionably costly, albeit sophisticated, interventions with the all-important appreciation that this is an entirely realistic undertaking even in an under-resourced area of the globe.

Secondly, under these circumstances, outcome analysis is seen to approximate that from recognized reference centers in the field. This applies in the first three months with mortality largely related to the procedure itself. Then also, and of note, given the vagaries of southern Africa with migrant populations and civil unrest, the opportunity of serially observing these individuals for more than 35 years to provide a reliable long term reference point for overall cost effectiveness.

Thirdly, in the specific context of ex vivo T-cell depletion is the seminal observation that survival curves are stable and do not have late and previously unrecognized sequelae that differ from those generated in recipients exposed to conventionally used post-transplant immunosuppressive regimens. Thus there is no apparent impact of this unique immunosuppressive regimen on developing myelodysplasia or predisposing to chronic infections such as pulmonary tuberculosis so rampant in sub-Saharan Africa. It remains to be determined whether the slightly higher incidence of cytomegaloviral infections translates into any clinically important requirements other than the need for prompt recognition and proactive antiviral therapy. It is also momentarily unclear as to whether the impact of retroviral infections can be altered in the face of appropriate combination active retroviral therapy and this is the subject of ongoing study.45 Currently the quality-of-life with the low incidence of graft versus host disease, in its various forms, is gratifying.

Fourthly veno-occlusive disease has not been seen despite the use of intravenous busulphan. Furthermore peripheral blood stem cell mobilization with G-CSF has been efficient with only relatively minor side effects of backache and bone pain in some donors. No difference can be seen when bone marrow is compared to peripheral blood as source of the graft. Also, and of relevance, is that no serious adverse reactions46 have thus far been noted in donors despite which informed consent has been altered to include extended surveillance both among siblings and matched unrelated volunteers.
through each referring registry.²,¹²⁷

Fifteenth, immunohaematopoietic stem cell grafting including collaboration on a worldwide basis is entirely realistic in this country given that donations can be imported or exported with due observance to rules on moving human tissue between countries.²,¹²⁷ This, in turn, is relevant and underlines the importance of our having established the South African Bone Marrow Registry²⁰⁴ that functions interchangeably with others throughout the world including in the United Kingdom, Europe¹⁰⁹ and the American National Marrow Donor Program.¹⁰⁸ Logically such a network can provide an impartial repository to monitor country-wide activity with the further attraction of a database upon which to plan national protocols, co-operative clinical trials and research studies.

Conclusion

These procedures carried out in a properly constituted and dedicated unit continues to generate results that are comparable to those from established first world reference centres. The low transplant related mortality, rejection rate and graft-versus-host disease, previously reported are confirmed. The benefits of what is clearly a center effect cannot necessarily be extrapolated to similar interventions performed outside designated and monitored academically orientated facilities. In Third World countries, given current parlous financial climate and rampant retroviral epidemics, allocation of restricted resources for this purpose is rationally most sensible when limited to established, audited and accredited teams. Such a stance rests heavily on publication of outcome to justify ongoing support. It will also direct focus to actual achievement in contrast to the currently favoured incentive-driven preferred providers.

Acknowledgments

We recognise the courage of our patients and their families. Thanks to the staff in the Joanne Porter Apheresis and Bone Marrow transplant units, the Sunflower Haematology ward and the outpatient clinic for their high level of professionalism and care.

Supported by the Haematological Research and Education Trust and Chairman’s Fund of the Anglo-American Corporation with grants from The Anthony Taberer, Louis Shill and Margaret Ward Foundations (Lucille Wood and Peter Jacobs): Manufacture of the Campath antibodies was supported by the UK Medical Research Council, the EP Abrahams Trust, Millennium Pharmaceuticals Inc (formerly LeukoSite Inc) and TolerRx Inc. Thanks are due to all members of the team at the Therapeutic Antibody Centre Oxford, notably Pru Bird, Patrick Harrison, Eleanor Berrie, Emma Belam and Tony Gallagher. Tissue typing was via the South Africa Bone Marrow Registry and we recognise the excellence of that internationally accredited immunogenetics group.

Some of the work referred to in this thesis was an Honours project for BSc in Actuarial Science awarded to Jonathan Haveman.

We thank Mr Pedro Abrunhosa for help with the graphics. Christine Dolling for the bibliographic review and Natasha Trueman typed the manuscript: we record appreciation and thanks to our research staff.

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null


CHAPTER 8

CONCLUSION

THE PAST – PRESENT – AND FUTURE
Orientation

The analysis and reporting of this long-running research project is considered, with every possible humility, as but one additional step in continuing to import relevant knowledge into our country as a basis for further development of an established, clinically applicable and multidisciplinary technology. In reaching this point each of the four goals originally identified have been largely realised. Specifically creating a suitable experimental environment to set-up the haematology laboratory, then transferring the methods to relevant and standardised clinical protocols, penultimately ensuring participation in scientific advances exploring innovative therapies and, finally, maintaining accepted international academic and intellectual standards to comply with registry participation and accreditation.

Concerns were expressed in the Lancet this year, on the behalf of the Academy of Science of South Africa, about clinical research in our country\(^2\rightarrow7\). For this reason a compelling argument can be mounted to invigorate immunohaematopoietic stem cell transplantation by capitalising on sustained local achievement some of which carries with it strong worldwide recognition and collaboration.

To give credence to such a stance it is instructive to look back over the voluminous records meticulously collected and catalogued. The data is found to fall quite naturally into three interlocking, albeit consequential, segments.

The past dates from the mid to late 1960s recognising a need to introduce bone marrow transplantation into our country. This was realised by drawing on experience from many parts of the globe in defining technology and building the necessary infrastructure essential for a credible scientific programme. Much time and effort was invested in the animal model as well as establishing entirely new techniques including clonogenic assays. Inevitably there was a steep learning curve linking many different disciplines through an unusual esprit de corps best described as remaining perpetual students. International interaction anchored this growth with guidance offered from numerous other lands culminating in emergence of the first South African transplant centre.
The **present** accounts for most of these years given to constantly forging a multidisciplinary basic science and clinical research facility. The resulting matrix was sufficiently productive of good quality published and presented outcome studies to be allocated specific topics for investigation within major cooperative scientific groups. These included the innovative new method for immunosuppression with cyclosporine A and benchmark exploration of the Campath series of monoclonal antibodies leading to description of *ex vivo* use or in-the-bag technique. A distinct achievement rests on accountability for every consecutive procedure leading to more than three decades of unbroken accreditation by major registries worldwide. A further significant challenge was relocation of almost the entire prototype team from the University of Cape Town in Groote Schuur Hospital to a newly developed academic centre in the private sector accompanied by seamless transfer of all the long-standing links so carefully established within the worldwide transplantation community. Ever sensitive to the importance of designation at teaching hospital level was the privileged opportunity to qualify for satellite status in the Division of Clinical Haematology - Health Sciences Faculty at Stellenbosch University.

The **future** is projected along three interrelated axes. One is to consolidate status as a reference centre for new facilities being developed throughout this under resourced part of the world. The second to sustain intergroup contact to constantly upgrade and enhance local science and clinical treatment protocols. The third to bring clear focus to the spectrum of disturbed immunologic integrity that occurs during post-transplant reconstitution and the all-important concept of survivorship. Such phenomena are overshadowed by the acquired immunodeficiency disease in sub Sahara adding an undefined new dimension to the role of these therapies in unique lymphoproliferative and myelodysplastic disorders.

**Organisation of research data**

Using this time-frame as the template only carefully selected publications have been grouped by dominant topic into consecutive chapters. Within each, as well as throughout the summary of the work in its entirety, contribution from local colleagues is readily and gratefully acknowledged. Also recognition is unhesitatingly given, with considerable appreciation, to the still active participation in a number of international study groups.
The weight of direct involvement in any publication identified by asterisk where the investigator not included in writing committee but interactive with the registry or group to variable extent.

Similarly, although in a slightly more personal way, to identify those who continue to make up The Haem Team ranging from janitorial and clerical staff through research typists and librarians to medical or nursing and other paramedical colleagues. The citation of published work is not exhaustive but rather critically chosen to keep within the confines of the research theme. The conclusion then briefly recapitulates the major positive findings allowing a strong closing statement to place in perspective new contributions outlined in this work as an anchor upon which to build in the future.

The stimulus to study and introduce bone marrow transplantation in South Africa

In keeping with long established European traditions haematology was based in University Pathology Departments contrasting with the alternative acceptance of a vital clinical component pioneered largely in North America. Change was as spectacular as it was acute following the first human heart transplant by Professor Christian Neethling Barnard and his team. Tissue typing and the immunobiology of tissue and organ transfer created opportunities at the University of Cape Town that coincided with exposure to immunohaematopoietic stem cell allografting during Fellowship at the University of Washington in Seattle. With the honour of appointment as Professorial Head of the new autonomous Department of Haematology at this prestigious Institution the direction was established with the future clinical base to be in Groote Schuur Hospital.

Experimental haematology

Although much of the experience worldwide was based in a mouse model an alternative approach, using inbred strains of rabbits, was made possible through collaborative studies with Swiss scientists. All the methodology needed to harvest bone marrow and create a monocellular suspension that would be required in the human procedures meticulously standardised. From this point it was possible to move into the clinical programs.
Development of laboratory and clinical infrastructure

Once recovered the starting material was characterised in terms of cell numbers and immunophenotypic composition with flow cytometry in parallel with repopulating capacity using in vitro culture or clonogenic assays. Apart from internal quality control split sample studies were cross-checked at European and Canadian reference laboratories.

The necessary granulocyte and platelet infusions, neither available in South Africa at that time, predicated introduction of apheresis technology and establishment of dedicated donor panels initially from staff in our Department. The next step was to apply the methodology to separation of stem and progenitor cells from marrow-rich blood and extending this to recovery of the same population from peripheral circulation following parenteral stimulatory peptides. It now became pertinent to create a cryobiology section providing further options to the available range of graft sources. Harvest techniques in the human were refined in a number of cadaver exercises. Once all the requirements had been identified compliance needed to initiate the first procedure obtained from the Hospital and University Ethics and Research Committee.

Initially at new Groote Schuur a dedicated facility was engineered in association with experts from the Fred Hutchinson Cancer Institute in Seattle and built into the newly developed State Hospital and subsequently duplicated first in Wynberg and then in Constantiaberg Medi-Clinic. Implicit in such a transplant unit was committed involvement to all the specialities needed to effectively operate multidisciplinary, often daily, consultation from infectious disease, pulmonology, gastroenterology, dermatology and very particularly professional nurses and paramedical professionals including academic dieticians, psychologists and psychiatrists – the morning report. Central to effective coordination, data capture and maintaining records was establishment of a career structure for a Chief Professional Nurse designated as Haematology Coordinator and filled with great distinction by Sister Lucille Wood – in her own right a registered medical natural scientist. This interactive approach, introduced first at the University of Cape Town, continues as the standard being upgraded and refined. Interestingly the structure has recently been duplicated elsewhere in the country.
Acknowledging the benefits of constant education has been the creation of an exchange program with the University of Omaha in Nebraska for senior residents and, most notably, established investigators from the nursing Department.

Audit with registry participation and accreditation
In addition to case-by-case review of the initial patients transplanted it was immediately appreciated that such, if free standing, would be inadequate for acceptance into the international transplantation community. Accordingly, after a probationary period, every consecutive procedure was reported to the International and then the Autologous registries. This continues to the current day with all these activities consolidated in the Centre for International Bone Marrow Transplant Registration. Additional information is provided to The European Group for Blood and Bone Marrow Transplantation. On this basis and as result of random ongoing audit the original team continues with an uninterrupted record of accreditation extending beyond three decades and currently still active.

Cyclosporin A studies
With accumulating experience, confirmed from within all the participating groups, remained dual and very real problems of rejection and graft-versus-host disease as paramount side-effects to overcome. Collaboratively this unique undecapeptide of fungal origin was tested and found to be effective. However it did not abrogate either acute or chronic variants of reverse rejection. Current usage is limited to histoincompatible pairs and the subject of ongoing study.

Campath monoclonal antibodies and in-the- bag technique described
In an extension of endeavouring to blunt post-transplant side-effects the opportunity arose to collaborate with colleagues originally in Cambridge and subsequently relocated to the Dunn School of Pathology in Oxford. These ongoing studies are noted regularly by the Campath users group. Of particular significance is the generally accepted landmark study in which, as a result of experimental endeavours in our Department, these antibodies were used not in vivo to the patient but exposed to the incoming graft ex vivo or in-the-bag.
There resulted effective decrease in typical graft-versus-host disease which has been replaced by a *forme frust*. This variant is readily responsive to topical corticosteroids and, notably, not accompanied by any significant increase in cytomegaloviral infections or leukaemic relapse.

**Standardised protocol with reporting of adult and paediatric outcome**

Coordinated management has made possible, on the still active and continuing basis, declaration of outcome in respect of all results at two levels. Firstly freestanding scientific reports from this team in first-line and peer-reviewed journals. Notable here the survival curves approximate those occurring in major first world reference centres. Secondly acknowledgement for inclusion of relevant contributions in publications and presentations from international collaborators being particularly the Centre for International Bone Marrow Transplant Registration and the European Bone Marrow Transplant Registry.

**Designation as an international transplant, harvest and donor centre**

In addition to individual case reporting the overall status of the team, measured by stringent worldwide criteria, maintains approval and designation in each of these categories by registering authorities worldwide. Furthermore, in recognising the need as well as local difficulties in finding allograft donors where siblings are not available, initiated the concept and the successful establishment of the South African Bone Marrow Registry. This laboratory has now been registered for immunogenetics and enjoys regular interaction with reference centres including long running consultation with colleagues in Leiden.

**Focus on immunologic reconstitution and the concept of survivorship**

Considerable spin off is constantly becoming available from the main line treatment protocol with the established research programmes both locally and internationally. One of these is to focus on the immunologic aspects of these procedures and such is to be undertaken jointly with Professor Patrick Bouic in his Department at Stellenbosch University. The second, having benefited from the high profile at the recent South African Lymphoma Study Group meeting jointly with the German Hodgkin counterparts, drew crisp attention to the largely under represented and poorly reported, albeit sometimes subtle, side-effects.
These increase with time both quantitatively and qualitatively. Accordingly, to focus particular attention on this very important aspect of the whole range of such interventions, a formal study is now in progress with a PhD student registered at Stellenbosch University.

**Achievement as a basis for future direction in an under resourced South Africa**

Ripple effects from this research endeavour are seen in a number of actively expanding arenas.

Of major importance for resource allocation on a national basis is the concept of developing closer interaction between private and public sector. Although the impact of a national health insurance remains to be determined this may further compromise such future interaction. Whilst neutrality is important there is nevertheless a sense of responsibility to protect this important segment of medicine.

Secondly the repository of a secure and internationally accredited team is self-evident from the way in which a number of practices throughout the country are increasingly embarking on these procedures in one or other form.

Thirdly, and in this latter regard, is the strong encouragement for all such practitioners to seek accreditation - preferably international - with the understanding that this could perhaps be offered nationally. This type of advance could take into account changes that are likely to arise in legislation affecting the human tissues act and impacting on the broad ambit of transplantation with special focus as regenerative applications extend to exploring reparative options.

Fourthly, neither should the capacity to use these techniques in properly selected cases of solid tumour oncology and a range of autoimmune diseases be glossed over but here there would be strong emphasis for developing national coordinating programs.

Fifthly, while a modest leadership role has already been taken in documenting activity between the different centres it would, with deference, be hoped that such a common sense approach will receive the necessary endorsement from licensing authorities including the Department of Health.
Sixthly, and not to be overlooked, are alternative graft sources ranging from umbilical blood or cord transplants to amniotic fluid with thought needing to be given to centring this on National venture rather than random unsupervised, growing by default, in the private sector.

Penultimately there is a high priority to understand the impact of human immune deficiency virus infections, as they will certainly influence a whole new spectrum of lymphoproliferative disorders.

Finally many of these activities are seen as precedent creating important growth points that are best sustained in Fellowship training programs as have proven so successful in other parts of the world. Examples already demonstrable are gene rearrangement studies offering better insight and particular application to further document differences in geographical haematopathology. A positive step is interaction with Nebraska Medical School in Omaha extending the long-running lymphoma reclassification project.

It has been a pleasure and an enormous privilege to associate with a very wide range of local and international colleagues - all fellow students - over these many decades. Few could have asked for greater scientific, academic or intellectual fulfilment. Add to this the examples daily provided by patients and families one is left with an indelible legacy of what little has been possible and how much remains to be done.

A fitting quote on which to bring all this experience together might therefore be:

*Tot Faciendum Parum Factum*

So much to do - so little done.
health system and its people if the government were to continue on its previous path. We hope that this Series and the accompanying comments will serve as the basis for serious discussion and ultimately decisive action to improve health outcomes for South Africans.

It is encouraging that the new Health Minister, Aaron Motsoaledi, a physician with long experience in provincial politics, touched on many of these fundamental issues in his budget speech on June 30. He will need to show financial and political commitment in the face of the international economic crisis and additional destabilising effects, such as an HIV epidemic and the effects of climate change. A strong, stable, and equitable South African health system with tangible outcomes should be the most important legacy of this new government.

Three new strategies are needed to form the basis for change. First, to establish a dedicated and strengthened—in skills and numbers—health workforce for the public sector. Second, to ensure sustainable and equitable access to health services for all through the introduction of an affordable national health insurance system. And third, to give leadership and managerial positions to those who are most competent and who are not afraid of being held accountable. There is no place for corruption, nepotism, or misguided political correctness. Only after these changes have been implemented can the real work begin and the current downward trajectory be reversed.

The South African people have shown extraordinary resilience during difficult times. The current leaders have survived apartheid, and often imprisonment, to fight for the future of their country. Civil society, with its strong voice, has brought about many important changes in health. South Africa is a young democracy with pride and hope, and above all with high expectations for a fair, equitable, and peaceful society. Its people deserve a healthy future.

Sabine Kleinert, Richard Horton
The Lancet, London NW1, UK
We thank Mickey Chopra, Hooosen Coovadia, Rachel Jewkes, Salim Abdool Karim, Joy Law, Fionnuala Maylor, Ashley Van Niekerk, and all others who have worked on this Series to make it possible.

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academically active, to inject their expertise into the service sectors, and to influence health delivery policies.3

As a result, many clinical researchers have been left with no option but to turn to the drug industry for the funding of those clinical trials in which the companies concerned have an interest, or to international donors who undertake large-scale but short-term to medium-term projects in South Africa, with local researchers drawn into international teams.4 Drug-industry investment is aimed predominantly at profitable areas, such as chronic diseases of lifestyle, mental illness, and allergy, whereas most of the external donor funding is directed at the local HIV and tuberculosis epidemics.

The serious decline in clinical research activity and capacity has prompted the Academy of Science of South Africa (ASSAf)4 to launch a consensus study designed to make recommendations on reinvigoration of clinical research in the country. An additional stimulus is the emphasis by the government in its 10-year science and technology plan on the development of new medicines and other biologically useful agents (“farmer to pharma”).4 A 14-member panel chaired by Bongani Mayosi of the University of Cape Town is expected to release an independently peer-reviewed report later this year.

Scholarly publication and the accompanying targeted dissemination of new information is an important, measurable indicator of activity in particular research areas.5 South Africa is one of many developing countries that produce only a tiny fraction of the world’s health research literature.6 Despite this shortage, the country dominates sub-Saharan Africa in terms of number of publications, and was for some time particularly productive, in world terms, in clinical medicine. Since the 1960s, health-related research has accounted for the largest proportion of South African literature in the indexed Thomson Reuters ISI Web of Knowledge system. An exhaustive study of South African research articles included in this system showed that 50% were in the medical and life sciences (the analogous figure for Africa overall was no less than 61%).7 Groote Schuur Hospital in Cape Town alone achieved a greater number of the 1% most-cited papers in the ISI system during 1981–91 than did all but two of South Africa’s higher education institutions.8

More recently, however, the share of total ISI-indexed papers from South Africa in clinical medicine has fallen by almost a fifth, from 0.59% in 1990–94 to 0.48% in 1996–2000, mostly caused by a failure to keep up with the rapidly growing total number of publications.9 Most South African ISI-indexed papers in clinical medicine are now published in international (foreign) specialty journals; the number of reports published in local journals, ISI-indexed or not, has substantially decreased in recent years. Articles describing randomised trials in the local flagship South African Medical Journal numbered 195 between 1976 and 1987, but only 92 between 1988 and 1997.10

The capacity to continue to publish articles in leading international specialty journals depends on survival mechanisms under conditions of public disinvestment. There are still a few South African groups with high standards in design and execution of clinical research projects, international collaborations, and effective training programmes. The lack of purposeful and coordinated planning and support makes them fragile and limits their number, however. The recent success of the national Department of Science and Technology in mobilising funding from the National Treasury for Centres of Excellence, research chairs, and major new equipment has been associated mainly with the natural sciences and engineering, which are the preserve of the National Research Foundation, itself controlled by the Department of Science and Technology. The Medical Research Council, which falls under the Department of Health, has failed so far to link itself to these important and highly effective investments. The Department of Health has also been unable to achieve its declared targets of spending on health research.11 Dysfunctional
Otamixaban in acute coronary syndromes

Anticoagulants are widely used to prevent recurrent ischaemic events in patients with acute coronary syndromes. For those who are managed medically, low-molecular-weight heparin (LMWH) or fondaparinux are often used instead of heparin because of the convenience of subcutaneous administration once or twice daily. By contrast, intravenous heparin or bivalirudin is preferred for patients with acute coronary syndromes who are undergoing percutaneous coronary intervention (PCI) because these agents have a rapid onset of action and a short half-life, and the dose can be titrated with point-of-care coagulation monitoring.

Otamixaban is a new direct-acting parenteral factor Xa inhibitor. Like heparin and bivalirudin, otamixaban has a rapid onset of action and a short half-life (table). Otamixaban produces a predictable anticoagulant effect, which obviates the need for routine coagulation monitoring, and unlike heparin or LMWH, the drug does not cause heparin-induced thrombocytopenia. Finally, with less than a quarter of the drug cleared by the kidneys, dose modifications are unlikely to be required in patients with mild or moderate renal impairment. On the basis of these characteristics, otamixaban has the potential to replace heparin in patients with acute coronary syndromes who are undergoing PCI.²

In The Lancet today, Marc Sabatine and colleagues report the results of the Study Program to Evaluate the Prevention of Ischemia with direct Anti-Xa inhibition in Acute Coronary Syndromes 1—Thrombolysis in Myocardial Infarction 42 (SEPIA-ACS1 TIMI 42). In this phase 2 dose-finding study, the investigators compared five doses of otamixaban (0.08 mg/kg bolus followed by infusions of 0.035, 0.07, 0.105, 0.14, or 0.175 mg/kg per h) with the combination of heparin plus eptifibatide for the prevention of major cardiovascular events in more than 3200 patients with non-ST-elevation acute coronary syndromes. More than 98% of patients received concomitant therapy with aspirin and clopidogrel, 99% had coronary angiography, and 63% had PCI. During the first 7 days, the rates of the primary efficacy outcome, a composite of death, myocardial infarction, urgent revascularisation, or bail-out glycoprotein IIb/IIIa use, were not significantly different across the five doses of otamixaban or with any of the otamixaban doses compared with heparin plus eptifibatide. However, the group who received the lowest dose of otamixaban was stopped early, and with this dose and the next lowest dose of otamixaban, the rates of thrombotic complications or use of bail-out glycoprotein IIb/IIIa inhibitor during PCI were at least two-fold higher than...
CHAPTER 9

SELECTED BIBLIOGRAPHY
Criteria for publications selected

In each of the sections, as noted on page 8 – Objectives – Title and Scope - quoted works fall into two broad categories. In the first – identified by an asterisk – the investigator had access to restricted protocols, privileged reports and ongoing trial data from registries but where citation is deemed relevant to this research project. No immediate role is claimed but rather acknowledges advice, help and collegial scientific as well as academic interchange.

In the second, all the remainder, project planning, direct participation, data analysis and full involvement in all stages is compliant with authorship and thus the criterion for inclusion.

The entire bibliography is sequential and tracks advances occurring throughout the world in the different and evolving aspects of immunohaematopoietic stem cell transplantation. Thus the arrangement is along these time lines, not segregated by geographical or methodological consideration such as graft source. The listing therefore documents the prospective evolution of the project over this thirty year period.

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Chapter 4 – Cyclosporin-A – The first local innovation


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Chapter 5 – Campath monoclonal antibodies – The second local innovation


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**Chapter 6 – Peripheral blood stem cell grafting – Transfer of evolving technology –**

The third local innovation


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*Chapter 8 – Conclusion – The past-present-future

CHAPTER 10

SUPPORTING CURRICULUM VITAE
ABRIDGED CURRICULUM VITAE - 2010

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      South African Medical and Dental Council

1960: Physician
      South African Medical and Dental Council

1961: Medical Practitioner
      General Medical Council: United Kingdom

1996: Pathologist - Haematology as Speciality
      General Medical Council: United Kingdom

1998: Clinical Haematologist
      Health Professions Council of South Africa

RESEARCH BURSARIES AND FELLOWSHIP

1961-1963: Senior Research Bursar,
            CSIR Iron and Red Cell Metabolism Unit, Department of Medicine,
            University of the Witwatersrand, Johannesburg

1967-1969: Senior Research Fellow in Haematology,
            Department of Medicine, University of Washington, Seattle,
            Washington

PROFESSIONAL QUALIFICATIONS - COLLEGES

1960  Membership, British Medical Association
1966  Fellowship, The College of Physicians, Surgeons and Gynaecologists of
      South Africa
      The College of Medicine of South Africa
      Fellowship, The Royal Society of Medicine, UK
1967  Membership, International Society of Haematology
1973  Membership, The American Society of Hematology
1974  Membership, New York Academy of Sciences
1977  Membership, The International Society of Experimental Hematology
      Fellowship, The American College of Physicians
      Membership, The Royal Society of South Africa
1978  Membership, The American Association for Cancer Research
1979  Membership, The American Society of Clinical Oncologists
      President-Elect, The Southern African Society of Haematology
58          The European Bone Marrow Transplantation Group
1982  Fellowship, The Royal College of Pathologists, UK
1983  Membership, The Scientific Advisory Board, Immunology and
      Haematology Research
      Membership, The Council of Biology Editors
      Membership, International Society of Thrombosis and Haemostasis
      Honorary Advisor, International Division, The American Biographical
      Institute
      Membership, The New York Academy of Sciences
      Membership, The Southern African Transplantation Society
      Membership, The European Bone Marrow Transplantation Group
      Membership, The European Society for Haemapheresis
Director, African and Asian Chapter, The European Society for Haemapheresis
Membership, Biological Standardisation Committee, Medicines Control Council

1985 - Membership, The College of American Pathologists
1986 - Foundation Member, South African Society of Medical Oncology
1986 - Fellowship, The Royal Society
Fellowship, International Research into Science and Technology
Counsellor, European and African Division
Board of the Council, International Society of Haematology
Fellowship, American Society of Clinical Pathologists
Director, Board of Directors for America, African Countries, Asia and Pacific Countries, European Society for Haemapheresis

1987 - Membership, World Apheresis Association
1991 - Foundation Member, South African Society of Medical Oncology
1991 - Fellow, International Biographical Association
1992 - Foundation Member, South African Lymphoma Study Group
1992 - Foundation Member, South African Bone Marrow Registry
1994 - Fellowship, Academy of Haemostasis and Thrombosis
1994 - District Counsellor for Africa on the 1994-1996 WAA Board of Directors
1995 - Tygerberg Comprehensive Lymphoma Study Group
2006 - Sub-Saharan Africa Lymphoma Consortium
2006 - Life Fellowship, The Royal Society of Medicine
2008 - International Fellow, American Society of Clinical Pathologists
2008 - Emeritus Member, American Society of Haematology
2008 - Emeritus Member, European Society for Haemapheresis and Haemotherapy
2008 - Emeritus Member, International Academy of Clinical and Applied Thrombosis/Haemostasis
2008 - Emeritus Member, International Society for Interferon and Cytokine Research
2009 - Emeritus Member, The Royal College of Pathologists
2009 - Emeritus Member, The Royal College of Physicians Edinburgh
2009 - Emeritus Member, American Society for Blood and Marrow Transplantation
2009 - Emeritus International Fellow, The College of American Pathologists

POSTGRADUATE EXPERIENCE

1961-1963: Part-time Casualty Officer,
Senior Research Bursar, CSIR, Iron and Red Cell Metabolism Unit,
Department of Medicine, University of the Witwatersrand, Johannesburg

1963-1967: Clinical Tutorial Registrar
Department of Medicine, Johannesburg General Hospital and
University of Witwatersrand

1967-1969: Senior Research Fellow in Haematology
Instructor in Medicine
University of Washington
Director, Division of Haematology, Department of Laboratory Medicine,
University Hospital and King County Medical Centre
University of Washington,

University of the Witwatersrand and Johannesburg General Hospital.
1972-2010: Chief Specialist – Groote Schuur Hospital Teachings Group
1995 - 2010: Consultant Physician and Haematologist – Constantiaberg Medi-Clinic

**ACADEMIC APPOINTMENTS**

1972-1994: Foundation Professor of Haematology, University of Cape Town
Chief Specialist and Consultant Haematologist, Groote Schuur Hospital Teaching Group, Cape Town
Director, University of Cape Town Leukaemia Centre
Rotating Chairman, Division of Pathology, University of Cape Town

1995-2010: Emeritus Professor of Haematology
University of Cape Town
Honorary Consultant
Groote Schuur Hospital Teaching Group

1996-2003: Honorary Professor of Haematology
University of Stellenbosch
and
Tygerberg Academic Hospital

2001-2010: Professor of Internal Medicine, College of Medicine,
University of Nebraska Medical Centre

2003-2010: Foundation Professor and Head, Division of Clinical Haematology –
the Department of Internal Medicine
Stellenbosch University and Tygerberg Academic Hospital

2010: Honorary Professor, Division of Haematopathology –
the Department of Pathology
Stellenbosch University and Tygerberg Academic Hospital

2010: Honorary Professor, Department of Internal Medicine

**CONCURRENT APPOINTMENTS**

1995-1997: Director
Haematology Clinic and Bone Marrow Transplant Centre
Wynberg Hospital, Cape Town

1998-2010: Director and Head
Department of Haematology and Bone Marrow Transplant Unit
Constantiaberg Medi-Clinic, Plumstead, Cape Town
incorporating
The Searll Research Laboratory for Cellular and Molecular Biology

**INTERNATIONAL APPOINTMENTS**

1984: The European Society of Haemapheresis

2000: The Scientific Advisory Board for Immunology and Haematology Research
2008: Honorary Consultant: Stem Cell and Immunotherapy Unit, Barbados West Indies “The Peter Jacobs Stem Cell Suite”

AWARDS AND PRIZES INCLUDE

1956-1958: Ernest Oppenheimer Memorial Travelling Scholar
1956-1958: Chalarick Solomon Memorial Scholar
1959: David Lurie Memorial Prize in Surgery
       Medical Graduates Prize in Medicine
1967-1968: Eli Lilly International Fellowship
1968-1969: The University of Washington Research Fellowship
1970: S.L. Sive Memorial Travelling Fellowship
1990: The first 500, The International Biographical Centre Cambridge, England
1991: Andries Blignault Memorial Medal
       Glaxo Research Award
2009: Centenary Medal, Die Suid-Afrikaanse Akademie vir Wetenskapp en Kuns

ACADEMIC HONOURS INCLUDE – FROM 1970

Counsellor, European and African Division Board of the Council

Director, African and Asian Chapter, The European Society for Haemopheresis

Director, Board of Directors for the Americas, African Countries, Asia

District Councillor for Africa 1994-1996 WAA Board of Directors

Fellow, International Biographical Association

Honorary Advisor, International Division, the American Biographical Institute

President, Southern African Society of Haematology

Fellowship, International Research into Science and Technology

Visiting Professor to many prestigious academic institutions in Europe and the United States of America

Chairmanship to major haematologic and research meetings

Invitations to deliver plenary sessions, seminars and lectures at international meetings

INVITED INTERNATIONAL PAPERS, FACULTY LECTURES & SEMINARS

Over 100 original research presentations
29 Organised symposia and congresses (1978-2002)
12 Non-medical lectures (1976-1994)
INTERNATIONAL RESEARCH COLLABORATION

Study of C. parvum immunotherapy in acute leukaemia (Wellcome Research Laboratory)
Epipodophyllotoxin VP16-213 as an anti-cancer agent (Swiss and American Research Groups)
Polycythemia Vera Study Group
SASIB Breast Cancer Study Group
International Acute Aplastic Anemia Study Group
CIBMTR – Center for International Blood and Marrow Transplant Research
NMDP – National Marrow Donor Program
IMWG – International Myeloma Working Group
Autologous Bone Marrow Transplantation Registry
European Group for Blood and Marrow Transplantation Registry
International Study Group for Interferon in Chronic Granulocytic Leukemia
AML Collaborative Group, Oxford
ALL Group, Oxford
Campath Users’ Group, Oxford
World Apheresis Association
The Non-Hodgkin’s Lymphoma Classification Project

EXAMINER

15 Post-doctoral theses
15 Research theses supervised

EDITORIAL BOARDS

Experimental Hematology
Journal of Clinical Apheresis
Medicine International – Overseas Adviser
Immunology and Hematology Research
Bone Marrow Transplantation
Editor-at-large in the field of Haematology for Marcel Dekker
Apheresis Transfusion Science
Hematology – Associate Editor
Clinical Lymphoma
Hematology/Oncology and Stem Cell Therapy

BIBLIOGRAPHY INCLUDES

2 Theses – MD and PhD
3 Medical books
30 Non-medical books and diaries
44 Chapters in books and encyclopaedias
244 Original scientific publications in peer-reviewed journals
109 Major publications from international collaborative study groups reviewed journals
100 Preliminary communications and letters
327 Abstracts in reviewed journals & presentations at scientific meetings
213 Full length reviews and editorials
8 Miscellaneous publications
Presently in press are numerous research publications and abstracts
CURRENT INTERESTS OUTSIDE MEDICINE INCLUDE

Golf, tennis, gym fitness programs, motor mechanics, photography, hiking